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13. ABSTRACT *(Maximum 200 words)*

Using the procedures described in this report, we were able to work sequentially or simultaneously on development, validation and characterization of assays for WR 6026 (and its metabolites, WR 211789 and WR 254421), mefloquine (and its metabolite, WR 160972), *p*-aminoheptanophenone (and related compounds), WR 242511, halofantrine (and its metabolite, WR 178,460, and their stereoisomers), chloroquine (and its metabolites, monodesethylchloroquine and didesethylchloroquine), WR 243,251, WR 238,605, quinine, doxycycline, and artelinic acid. Work on routine analyses of biological specimens during this period was performed for studies that required determination of concentrations of WR 238,605, mefloquine, chloroquine (and its metabolites, monodesethylchloroquine and didesethylchloroquine), doxycycline, halofantrine (and its metabolite, WR 178,460), and quinine. We worked on demonstrating sensitivity, specificity, linearity, lack of interferences, accuracy, and reproducibility of the analytical method, describing the extent of recovery for the method, and reporting on the stability of compounds of interest in specimens during storage and drug analysis to provide documentation in support of Investigational New Drug (IND) submissions to the Food and Drug Administration (FDA).

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FOREWORD

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INTRODUCTION

Introduction

This report describes technical work accomplished and information gained in performance of contract number DAMD17-97-C-7058, titled "Analysis of Investigational Drugs in Biological Fluids - Method Development and Analysis of Pre-Clinical and Clinical Samples," for the US Army Medical Research and Development Command (USAMRDC). Using the experimental procedures described in this report, we maintain the capability to complete projects on up to one new compound (for which no method is described in the literature) and up to two compounds (for which methods are described in the literature) per year in terms of method development, validation, and characterization. We demonstrate sensitivity, specificity, linearity, lack of interferences, accuracy, and reproducibility of the analytical method, describe the extent of recovery for the method, and report on the stability of compounds of interest in specimens during storage and drug analysis. Validation of sensitive and specific analytical methods follow procedures described in the Analytical Section Procedural Manual, Procedure 2D-3.5, "Procedure for Validation" and earlier versions. Methods developed are such that a single technician can complete at least 15 clinical samples in one day. These methods are robust and portable enough to be transported to other laboratories. Within our routine analysis laboratory, we maintain the capability to assay up to 3,000 samples per year. Routine sample analysis will be performed in accordance with applicable procedures described in the Analytical Section Procedural Manual, Procedure 2D-4.5. "HPLC Run Setup" and Procedure 2D-10.3. "LC/MS/MS Run Setup" and earlier versions. We have sufficient equipment and personnel to develop several candidate agents simultaneously and to be able to respond to changing priorities. We prepare and submit required reports in accordance with the contracted schedule.

Purpose of the Present Work

Work on development and/or validation of analytical methodologies during the current contract focused on assays for WR 6026 (and its metabolites, WR 211789 and WR 254421), mefloquine (and its metabolite, WR 160972), *p*-aminoheptanophenone (and related compounds), WR 242511, halofantrine (and its metabolite, WR 178,460, and their stereoisomers), chloroquine (and its metabolites, monodesethylchloroquine and didesethylchloroquine), WR 243,251, WR 238,605, quinine, doxycycline, and artelinic acid. Work on routine analyses of biological specimens during this period was performed for studies that required determination of concentrations of WR 238,605, mefloquine, chloroquine (and its metabolites, monodesethylchloroquine and didesethylchloroquine), doxycycline, halofantrine (and its metabolite, WR 178,460), and quinine.

For many years our research group has been actively involved in the development of analytical methods to assay for drug substances in biological fluids for pharmacokinetic, bioavailability, drug metabolism and drug

monitoring studies. This report describes the approach we took to develop sensitive (picograms per milliliter of biological matrix), specific and quantitative analytical methods to support pharmacokinetic and bioavailability studies of candidate chemical warfare antidotes, antiparasitic drugs, radioprotectants and anti-infectious disease drugs.

In addition, routine analyses of biological specimens to support pharmacokinetic and bioavailability studies as part of preclinical and clinical investigations undertaken for the purpose of new drug development were performed as a significant adjunct to method development objectives. Within our routine analysis laboratory, we maintain the capability to assay up to 3,000 samples per year for this contract.

There are many reasons for the U.S. military to develop various new drugs to protect or to treat soldiers confronted with the hazards of the modern battlefield. Like any pharmaceutical company, however, the military has to provide documentation in support of Investigational New Drug (IND) submissions to the Food and Drug Administration (FDA). Therefore, a great deal of work involving animal studies, preclinical and clinical trials, toxicity, metabolism and formulations must be carried out before a drug can be tried in the field. All of these studies depend on the adequacy of the analytical method for the particular compound. The route of administration and the dosage form are not necessarily the same in the field as in the clinic. For example, pyridostigmine is given prophylactically in the field, but the dose and route of administration are different for the treatment of myasthenia gravis or in anesthesiology.[1] Since military personnel are constantly involved in areas where they can be infected by parasites, including tropical or subtropical zones with drug-resistant forms, the U.S. Army needs to organize programs so that highly active and more effective new drugs can be discovered. These types of programs are generally ignored by private industry due to limited markets and profits.

This contract has offered us an interesting and stimulating challenge to utilize and extend our considerable capabilities to conduct method development and routine analysis in support of pharmacokinetic and bioavailability studies. Our participation in this contract was possible by virtue of the experience and expertise of our staff in the area of pharmacokinetics, which requires assurance of extensive and rigorous internal and external analytical quality. As a result of our extensive involvement in these analytical programs, the staff members working on this project are the best in the field and have acquired a broad range of experience in the analysis of organic compounds in diverse media.

Background of Previous Work

Studies conducted over the 13 years prior to contract DAMD17-97-C-7058 under previous contracts including DAMD17-92-C-2028, DAMD17-86-C-6150, DAMD17-85-D-0008, and DAMD17-83-C-3004 are listed in Tables 1 (study reports) and 2 (routine analyses reports).

TABLE 1: PREVIOUS STUDIES

Report No.	Report Date	Report Title	Test System	Test Article	Lower Limit of Quantitation
01	8/26/83	Analytical Procedure for the Determination of WR 6026 in Plasma	plasma plasma blood blood	WR 6026 WR211,789•2HCl WR 6026 WR211,789•2HCl	6.44 ng/ml 8.00 ng/ml 6.44 ng/ml 8.00 ng/ml
03	1/22/85	High Pressure Liquid Chromatography (HPLC) of Pyridostigmine in Plasma	plasma	Pyridostigmine	1.4 ng/ml
04	8/23/85	Ion-Paired Liquid Chromatographic Method for the Analysis of Halofantrine (WR 171,669) and its Putative Metabolite, WR 178,460, in Blood and Plasma	plasma plasma blood blood	halofantrine WR 178,460 halofantrine WR 178,460	0.900 ng/ml 1.40 ng/ml 0.900 ng/ml 1.40 ng/ml
05	7/21/86	High Pressure Liquid Chromatography (HPLC) of Pyridostigmine in Plasma Using Silica Gel Column and an Aqueous Mobile Phase	plasma	Pyridostigmine	1.39 ng/ml
06	1/8/88	High Pressure Liquid Chromatography (HPLC) of Mefloquine in Plasma	plasma	Mefloquine	10.0 ng/ml
07	1/12/88	High Pressure Liquid Chromatography (HPLC) of Pyridostigmine in Urine	urine	Pyridostigmine	13.7 ng/ml
08	9/23/88	High Pressure Liquid Chromatography (HPLC) of Physostigmine in Plasma with Ultraviolet Detection	plasma	Physostigmine	1 ng/ml
09	9/12/88	Quantitation of Physostigmine & Eseroline in Plasma by HPLC with Fluorescence Detection	plasma plasma	Physostigmine eseroline	0.1 ng/ml 0.1 ng/ml
10	9/14/89	Quantitation of WR 6026 (Free Base) in Plasma & Blood by HPLC	plasma blood	WR 6026 WR 6026	0.980 ng/ml
11	9/28/89	Quantitation of WR 2721 in Plasma by HPLC with Electrochemical Detection	plasma	WR 2721	0.100 µg/ml

TABLE 1: PREVIOUS STUDIES

Report No.	Report Date	Report Title	Test System	Test Article	Lower Limit of Quantitation
12	11/14/89	Quantitation of WR 3689 in Plasma by HPLC with Electrochemical Detection	plasma	WR 3689	0.0990 µg/ml
13	11/17/89	Quantitation of WR 238605 by HPLC	plasma blood	WR 238,605 WR 238,605	0.815 ng/ml 1.91 ng/ml
13	10/28/94 final report	Supplement I: Quantitation of WR 238605 as Free Base in Rat Plasma by HPLC and Fluorescence Detection	rat plasma	WR 238,605	2.00 ng/ml
13	5/16/96 (accepted as final 3/3/98)	Supplement II: Quantitation of WR 238605 as Free Base in Dog Plasma by HPLC and Fluorescence Detection	dog plasma	WR 238,605	1.00 ng/ml
14	8/29/89	Quantitation of Mefloquine (f.b.) in Plasma by HPLC, Extract. Meth	plasma	Mefloquine	8.00 ng/ml
15	12/19/90	Quantitation of Ribavirin and WR 249,992 (f. b.) in Plasma by HPLC with C18 Bonded Silica Gel Columns and Acidic Aqueous Mobile Phases	plasma plasma	Ribavirin WR 249,992	20 ng/ml 10 ng/ml
16	Canceled	β-arteether project canceled		WR 255663	
17A	4/25/90 final	Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Plasma and Blood by HPLC with a Silica Gel Column and an Aqueous Mobile Phase	human plasma plasma blood blood	halofantrine WR 178,460 halofantrine WR 178,460	0.960 ng/ml 0.928 ng/ml 0.960 ng/ml 0.928 ng/ml
17B	12/13/95 final as amended 4/26/96	Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Plasma and Blood by HPLC with a Silica Gel Column and an Aqueous Mobile Phase	human plasma plasma blood blood	halofantrine WR 178,460 halofantrine WR 178,460	2 ng/ml 2 ng/ml 0.960 ng/ml 0.928 ng/ml
17B	1/23/98 final report	Supplement I: Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Rat Perfusate by Precipitation and HPLC with a Silica Gel Column and an Aqueous Mobile Phase	rat perfusate	Halofantrine WR178460	0.520 µg/ml 0.510 µg/ml

TABLE 1: PREVIOUS STUDIES

Report No.	Report Date	Report Title	Test System	Test Article	Lower Limit of Quantitation
17B	1/28/98 final report	Supplement II: Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Rat Perfusate by Extraction and HPLC with a Silica Gel Column and an Aqueous Mobile Phase	rat perfusate	Halofantrine WR178460	10.4 ng/ml 10.2 ng/ml
17B	1/28/98 final report	Supplement III: Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Rat Bile by Precipitation and HPLC with a Silica Gel Column and an Aqueous Mobile Phase	rat bile	Halofantrine WR178460	0.416 µg/ml 0.408 µg/ml
17B	1/28/98 final report	Supplement IV: Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Rat Bile by Extraction and HPLC with a Silica Gel Column and an Aqueous Mobile Phase	rat bile	Halofantrine WR178460	20.4 ng/ml
17B	1/28/98 final report	Supplement V: Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Rat Liver by Precipitation and HPLC with a Silica Gel Column and an Aqueous Mobile Phase	rat liver	Halofantrine WR178460	0.540 µg/ml 0.540 µg/ml
18	Status report: 7/31/91	Quantitation of WR 6026 and WR 211,789 (WR 6026 Metabolite) in Plasma and Blood by HPLC with a Silica Gel Column and an Aqueous Mobile Phase	plasma blood	WR 6026 WR 211789	0.980 ng/ml 1.21 ng/ml
19	Status report: 1/14/92	Tentative title: Quantitation of Mefloquine and its Metabolite, WR 160972 in Biological Fluids	plasma blood	mefloquine WR 160972	7.36 ng/ml -
20	7/27/94 final report	Quantitation of Artelinic acid in Plasma by HPLC with a C18 Bonded Column	human plasma	Artelinic Acid	4.96 ng/ml
21	Validation complete	Tentative title: Quantitation of *p*-Aminoheptanophenone, *p*-Aminooctanophenone, and *p*-Aminopropiophenone in Dog Plasma by HPLC	dog plasma	PAHP PAPP PAOP	4.08 ng/ml 4.04 ng/ml 4.16 ng/ml

TABLE 1: PREVIOUS STUDIES

Report No.	Report Date	Report Title	Test System	Test Article	Lower Limit of Quantitation
22	7/18/94 in revision	Quantitation of WR 6026, WR 211,789, and WR 254,421 (as Free Bases) in Human Urine By HPLC	human urine	WR 6026 WR 211,789 WR 254,421	5.17 ng/ml 5.09 ng/ml 45.4 ng/ml
23	4/29/96 final report	Quantitation of Primaquine (Free Base) and its Carboxylated Metabolite in Human Plasma by HPLC and Ultraviolet Detection	human plasma	Primaquine WR 249725	28.5 ng/ml 20.0 ng/ml
24	10/21/97 final report	Quantitation of Paromomycin and Gentamicin in Human and Rat Plasma by HPLC	human plasma rat plasma	Gentamicin Paromomycin Gentamicin Paromomycin	0.1 µg/ml 0.1 µg/ml 0.1 µg/ml 0.1 µg/ml
25	11/22/95 final report as amended 3/29/96	Quantitation of Pyridostigmine (as Free Base) in Human Plasma By HPLC with a Silica Gel Column and an Aqueous Mobile Phase	human plasma	Pyridostigmine	1.53 ng/ml
26	12/12/96 final report	Quantitation of WR 242511 (as Free Base) in Human and Dog Plasma By HPLC with a Silica Gel Column and an Aqueous Mobile Phase	human plasma dog plasma	WR 242511 WR 242511	4.00 ng/ml 4.00 ng/ml
27	12/17/97 final report	Quantitation of WR 238605 *R&S* Enantiomers (as Free Bases) in Human Plasma by HPLC	human plasma	*R* WR 238605 *S* WR 238605	5 ng/ml 5 ng/ml
28	Draft in preparation	Tentative title: Quantitation of R&S Isomers of Halofantrine and WR 178,460 in Human Plasma by HPLC	human plasma	Halofantrine WR 178,460	
29	8/22/97 status report	Validation of a LC/MS/MS Method for the Determination of Chloroquine and Monodesethyl-chloroquine in Human Blood Samples	human plasma	Chloroquine Desethyl-chloroquine	20 ng/ml 20 ng/ml
30	In validation	Tentative title: Quantitation of WR 243251 in Human Plasma by LC/MS/MS	human plasma	WR 243251	1 to 5 ng/ml

TABLE 1: PREVIOUS STUDIES

Report No.	Report Date	Report Title	Test System	Test Article	Lower Limit of Quantitation
31	Draft in preparation	Tentative title: Quantitation of WR 238,605, Mefloquine, Chloroquine, Quinine and Doxycycline in Dog Plasma by LC/MS/MS	dog plasma	WR 238605 Mefloquine Chloroquine Quinine Doxycycline	
32	Draft in preparation	Tentative title: Quantitation of WR 238,605 in Human Plasma and Small Volume in Human Blood by LC/MS/MS and	human plasma blood	WR 238605	
33	In validation	Tentative title: Quantitation of Halofantrine and WR 178,460 in Human Plasma by LC/MS/MS	human plasma	Halofantrine WR 178,460	
34	In development	Tentative title: Quantitation of WR 254421 in Human Plasma by LC/MS/MS	human plasma	WR 254421	
35	In development	Tentative title: Quantitation of Artelinic Acid in Rat Plasma by LC/MS/MS	rat plasma	Artelinic Acid	

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis of Halofantrine Plasma Samples Obtained from Protocol Titled "The Relative Bioavailability of Three Oral Formulations of Halofantrine Hydrochloride"	10/23/87	Halofantrine WR 178,460	plasma plasma	971 971	AY 86-1D
Phase III Comparative Clinical Trial of 4 Regimens of Halofantrine and Chloroquine in Treatment of *P. falciparum* Malaria	6/27/90	Halofantrine WR 178,460 Halofantrine WR 178,460	plasma plasma blood blood	470 470 468 468	Hal/BP 89-7
Routine Analysis for Protocol Titled "Pharmacokinetics of Intravenous Halofantrine HCl"	12/18/90	Halofantrine WR 178,460 Halofantrine WR 178,460	plasma plasma blood blood sol'ns	434 434 429 429 20	Hal/PB 90-5
Routine Analysis for Halofantrine and WR 178,460 (as Free Bases) of Plasma Samples Obtained under the Protocol Titled "52-Week Chronic Oral Toxicity Study of WR 171,669 HCl (Halofantrine Hydrochloride) in Dogs" and "Analysis of Blood and Plasma to Verify in vitro Metabolism of Halofantrine and Partition of Halofantrine and WR 178,460"	7/16/91	Halofantrine WR 178,460 Halofantrine WR 178,460	plasma plasma blood blood	83 83 48 48	Hal/P 91-1&2
Routine Analysis of Plasma and Blood Samples for the Protocol Titled 'Disposition Kinetics of IV Desbutyl Halo-fantrine and the Effects of Gastric pH on the Bioavail-ability of Halofantrine-HCl'	2/4/92	Halofantrine WR 178,460 Halofantrine WR 178,460	plasma plasma blood blood dosing sol'ns	756 756 754 754 18	Hal/BP 91-3

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis for Halofantrine and WR 178,460 (as f.b.) of Plasma Samples Obtained under the Protocol Titled "Combined Chronic Toxicity and Oncogenicity Study of WR 171,669•HCl (Halofantrine Hydrochloride) in Rats"	9/23/91	Halofantrine WR 178,460	plasma plasma	118 118	Hal/P 91-4
Routine Analysis for Halofantrine and WR 178,460 (free bases) in Blood Samples Obtained for the Protocol Titled "Efficacy of Halofan-trine and Mefloquine in Treat-ment of Falciparum Malaria"	1/21/92	Halofantrine WR 178,460	blood blood	107 107	Hal/B 91-5
Routine Analysis of Mefloquine Plasma Samples obtained from Six Clinical Protocols from Thailand	2/25/88	Mefloquine	plasma	781	Mef/P 87-1B
Routine Analysis of Plasma Samples from Thailand for Mefloquine Concentrations	12/7/88	Mefloquine	plasma	388	Mef/P 88-11
Routine Analysis of Blood Samples for Mefloquine (Free Base) Concentrations	2/12/91	Mefloquine	blood	18	Mef/B 90-3
Routine Analysis of Physostigmine Plasma Samples from the Protocol Titled "Bioavail-ability and Pharmacokinetic Study of Physostigmine (WR 006570) in Beagle Dogs"	8/26/88	Physostigmine Eseroline	plasma plasma	198 198	Phy/P 88-5
Routine Analysis of Physostigmine Plasma Samples from the Protocol Titled "Bioavail-ability and Pharmacokinetic Study of Physostigmine (WR 006570) in Rhesus Macaques"	9/15/88	Physostigmine Eseroline	plasma plasma	196 196	Phy/P 88-6
Pilot Study - Analysis of Rat Plasma	9/14/88	Physostigmine Eseroline	plasma plasma	45 45	Phy/rP 88-8
Pilot Study - Analysis of Rat Perfusate	9/14/88	Physostigmine	perfus	37	Phy/rPr, 88-9 pilot

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Pilot Study - Analysis of Monkey Plasma	5/5/88	Physostigmine Eseroline	plasma plasma	8 8	Phy/mP, 88-10 pilot
Routine Analysis of Physostigmine (f.b.) and Eseroline (f.b.) Rat Plasma Bile, and Tube Binding Samples for Samples Obtained from WRAIR	1/18/90	Physostigmine Eseroline Physostigmine Eseroline	plasma plasma bile etc bile etc	92 92 20 20	Phy/rP, 89-6 pilot
Pyridostigmine in plasma (Israel)	5/14/86	Pyridostigmine	plasma	427	PY 85-4
"Pyridostigmine in plasma" (PY85-6-2 and PY85-6-3 combined) (Johns Hopkins, Millers)	7/3/86	Pyridostigmine	plasma	32	PY 85-6-4
Routine Analysis of Pyridostigmine Plasma Samples from Battelle Laboratories-MREF Protocol 27 (Battelle)	7/9/86	Pyridostigmine Pyridostigmine	plasmap lasma,bl ind	648 22	PY 85-2-3
Routine Analysis of Pyridostigmine Plasma Samples Obtained from Protocol Titled "Pharmacokinetics of Orally Administered Pyridostigmine and Comparative Bioavailability of Liquid and Tablet Formulations" (Subjects 1-30)	12/3/86	Pyridostigmine Pyridostigmine	plasma dose sol	1698 12	PY 85-1
"Pyridostigmine in plasma (Johns Hopkins,Sub.1-24)"	1/12/87	Pyridostigmine pyridostigmine	plasma infusate	969 23	PY 85-6-5
"Pyridostigmine in plasma (Johns Hopkins,Sub.1-24)"	3/12/87	Pyridostigmine Pyridostigmine	plasma dose sol	1102 27	PY 85-6-6B
Routine Analysis of Pyridostigmine Plasma Samples obtained from Protocol Titled "Development of a Primate Model for Evaluating Efficacy of Treatment Regimens Against Nerve Agent Poisoning:Part I: Pharmacokinetics of Pralidoxime Chloride, Atropine Sulfate, and Pyridostigmine Bromide" (PY85-3-1 through PY85-3-5 combined)	5/29/87	Pyridostigmine	plasma, monkey	439	PY 85-3-6B

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Battelle Rat Study Pyridostigmine in plasma (revised letter report)	7/28/87	Pyridostigmine	plasma, rat	102	none none
Battelle Dosing Sol'ns Pyridostigmine in plasma (revised letter report)	7/28/87	Pyridostigmine	dose sol	92	none none
Routine Analysis of Pyridostigmine Plasma Samples Obtained from Protocol Titled "14 day pilot dose range oral toxicity study in dogs" (Battelle)	7/30/87	Pyridostigmine Pyridostigmine	plasma dose sol	152 2	PY 85-2-2B
Pyridostigmine in plasma (Huntingdon dog)	9/30/87	Pyridostigmine	plasmadog	336	PY 85-5-3C
Routine Analysis of Pyridostigmine Plasma Samples obtained from Protocol titled "Comparative Bioavailability Studies of Pyridostigmine Bromide in Male Beagle Dogs " (31 July 1985) (Huntingdon dog)	10/7/87	Pyridostigmine	plasma, dog	324	PY 85-5C
Routine Analysis of Pyridostigmine Urine Samples from Protocol Titled "Bioavail-ability of Oral Pyridostigmine and Inhibition of Red Blood Cell Acetylcholinesterase by Oral and Intravenous Pyridostigmine"	2/3/88	Pyridostigmine	urine	110	Pyr/U 86-3B (renamed from AY86-3)
Routine Analysis of Pyridostigmine Plasma and Urine Samples from Protocol Titled "Pharmacokinetics and Pharmacodynamics of Sustained, Low-dose, Intravenous Infusions of Pyridostigmine"	2/24/88	Pyridostigmine Pyridostigmine Pyridostigmine	plasma urine infusate	498 72 24	Pyr/PU 87-2B

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis of Pyridostigmine Plasma Samples from the Protocol titled "Comparative Oral Bioavailability Studies of Two Wax Matrix Formulations of Pyridostig- mine Bromide in Male Beagle Dogs"	3/29/88	Pyridostigmine	plasma	341	Pyr/P 88-1
Routine Analysis of Pyrido-stigmine Plasma Samples from the Protocol titled "Safety, Tolerance, Pharmacokinetics and Pharmaco- dynamics of Single Oral Doses of Sustained Release Pyridostigmine in Healthy Men," dated 9/18/87	8/3/88	Pyridostigmine	plasma	558	Pyr/P 88-2
"Routine Analysis of Pyrido-stigmine Plasma Samples from the Protocol titled ""Safety, Tolerance, Pharmacokinetics and Pharmaco- dynamics of Single Oral Doses of Sustained Release Pyridostigmine in Healthy Men,"" dated Sept. 30, 1987"	8/2/88	Pyridostigmine	plasma	476	Pyr/P 88-3
Routine Analysis for Protocol Titled "Safety, Tolerance, Pharmacokinetics and Pharmacodynamics of Single Oral Doses of Pyridostigmine Administered by an Osmotic-Delivery Module (osmetr) compared to Pyridostigmine Syrup in Healthy Men"	5/12/89	Pyridostigmine	plasma	374	Pyr/P 89-2
Routine Analysis for protocol titled "Safety, Tolerance, Pharmacokinetics and Pharmaco dynamics of Single Oral Doses of a Commercial Formulation of Sustained-Release Pyridostigmine in Healthy Men."	5/16/89	Pyridostigmine	plasma	120	Pyr/P 89-3

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Safety, Tolerance, Pharmacokinetics and Pharmacodynamics of Intravenous Pyridostigmine and Oral Doses of Standard and Sustained-Release Pyridostigmine in Healthy Men & the Influence of Food on Oral Pyridostigmine Pharmacokinetics	11/13/90	Pyridostigmine	plasma	1250	Pyr/P 89-8
Routine Analysis for Protocol Titled "Effect of chronic pyridostigmine administration on heavy exercise in hot environments"	9/11/90	Pyridostigmine	plasma	37	Pyr/P 90-2
Routine Analysis for Protocol Titled "Effects of Pyridostigmine Pretreatment on Physiological Responses to Heat & Moderate-to Intense Exercise"	2/20/91	Pyridostigmine	plasma	142	Pyr/P 90-4
Routine Analysis for protocol titled "Simultaneous Modeling of WR238605 Succinate Pharmacokinetics and Methhemoglobin Pharmacodynamics in the Beagle Dog"	4/13/89	WR 238605 WR 238605	plasma blood	62 62	WR5/BP, 89-1 pilot
Routine Analysis for protocol titled "Simultaneous Modeling of WR238605 Succinate Pharmacokinetics and Methhemoglobin Pharmacodynamics in the Beagle Dog"	6/1/89	WR 238605 WR 238605	plasma blood	88 88	WR5/BP, 89-4 pilot
Routine Analysis for protocol titled "Simultaneous Modeling of WR238605 Succinate Pharmacokinetics and Methhemoglobin Pharmacodynamics in the Beagle Dog"	8/25/89	WR 238605 WR 238605	plasma blood	240 240	WR5/BP 89-5
Routine Analysis of WR 6026 Plasma Samples Obtained from Clinical Protocol Titled "Single-Dose Absorption and Pharmacokinetics of WR 6026 Hydrochloride in Healthy Subjects"	6/24/87	WR 6026	plasma	192	AY 86-2D

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis of Blood Samples from the Protocol Titled "Multiple-Dose Pharmacokinetics, Safety and Tolerance of WR 6026 Hydrochloride in Healthy Subjects"	4/21/89	WR 6026	blood	571	Wr6/B 88-7
Routine Analysis for WR 6026 and WR 211,789 (as f.b.) of Plasma Samples Obtained from WRAIR	2/13/91	WR 6026 WR 211789	plasma plasma	13 13	Wr6/PB 90-6
Routine Analysis for Halofantrine and WR 178,460 (as free bases) of Plasma Samples Obtained for the Initial Year of the Protocol Titled "Combined Chronic Toxicity and Oncogenicity Study of WR-171,669 (Halofantrine Hydrochloride) in Rats"	3/31/93 final report	halofantrine WR 178,460	rat plasma	118 118	Hal/P 91-4
Routine Analysis for Halofantrine and WR 178,460 (free bases) in Blood Samples Obtained for the Protocol Titled "Efficacy of Halofantrine and Mefloquine in the Treatment of Falciparum Malaria"	1/21/92 final report	Halofantrine WR 178,460	human blood	107 107	Hal/B 91-5
Routine Analysis for Halofantrine and WR 178,460 (as free bases) of Blood Samples Obtained under the Protocol Titled "Efficacy of Halofantrine and Mefloquine in the Treatment of Falciparum Malaria"	6/23/92 final report	mefloquine	human blood	107	Mef/B 91-5
Results assoc. with Hal/P 91-1	4/28/92 final report	halofantrine WR 178,460	dog plasma	29 29	Hal/P 91-6
Routine Analysis for Mefloquine (as Free Base) in Plasma Samples Obtained under the Protocol Titled "Evaluation of the Tolerance of Prophylactic Mefloquine Regimens"	3/1/93 final report	mefloquine	human plasma	660	Mef/P 91-7
Study continued as WR6/PU 93-1	8/3/92 data	WR 6026 WR 211,789	plasma	194 194	WR6/P 92-1

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis for Halofantrine and WR 178,460 (as f.b.) of Plasma Samples Obtained for the Second Year of the Protocol Titled "Combined Chronic Toxicity and Oncogenicity Study of WR-171,669 HCl (Halofantrine Hydrochloride) in Rats, HWA Study No. 193-558"	3/31/93 final report	halofantrine WR 178,460	rat plasma	154 154	Hal/P 92-2
Routine Analysis for WR 238,605 (as f.b.) of Blood and Plasma Samples Obtained for the Protocol Titled "Rising Single Oral Dose Safety and Tolerance Study of WR 238,605 Succinate"	2/6/95 final report	WR 238,605	human plasma, blood	893 74	WR5/PB 92-3
Routine Analysis for WR 6026, WR 211,789 and WR 254,421 (as free bases) in Plasma and Urine Samples Obtained under the Protocol Titled "Phase II Clinical Trial of Oral WR 6026 2HCl in Patients with Vis-ceral Leishmaniasis - Initial Dose Ranging for Efficacy, Safety and Tolerance"	3/12/93 data	WR 6026 WR 6026 WR 211,789 WR 254,421	human plasma, urine	117 68 68 68	WR6/PU 93-1
Routine analysis for Halofantrine and WR 178,460 (as free bases) of Plasma Samples Obtained for the Protocol Titled "Pharmacokinetics of a New Multiple Dose Halofantrine Regimen"	12/10/96 in review	halofantrine WR 178,460		642 642	Hal/P 93-2
No protocol	2/25/94 data	*p*-aminoheptanophenone	dog plasma	876	Pah/P 93-3
Routine Analysis for WR 238, 605 (as f.b.) of Plasma Samples Obtained for the Protocol Titled "Thirteen Week Oral Toxicity Study of WR 238,605 with a Thirteen Week Recovery Period in Dogs"	4/25/94 in revision	WR 238,605	dog plasma	330	WR5/P 93-4

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis for WR 238, 605 (as f.b.) of Plasma Samples Obtained for the Protocol Titled "Thirteen Week Oral Toxicity Study of WR 238,605 with a Thirteen Week Recovery Period in Rats"	1/20/94 final report as amended 11/4/96 accepted as final 7/27/98	WR 238,605	rat plasma	154	WR5/P 93-5
Routine Analysis for Primaquine and Carboxyprimaquine of Serum Samples Obtained for the Protocol Titled "Primaquine and Several Recommended Prophylactic Drugs against Falciparum Malaria: Field Trial II"	5/3/96 final report	primaquine carboxy metab	human serum	60	Pri/P 93-6
Routine Analysis for Halofantrine and WR 178,460 (as free bases) of Rat Liver, Bile and Perfusate Samples	10/28/94 final data	halofantrine	rat liver perfsate bile		Hal/lpb 93-7
Routine Analysis for WR 238,605 (as free base) Human Plasma and Blood Samples Obtained for the Protocol Titled "Pharmacokinetics, Pharmacodynamics, Safety and Tolerance of a Single Oral Dose of WR 238605 Succinate"	9/16/94 final data	WR 238,605	human plasma blood	120 120	WR5/PB 93-8
Routine Analysis for *p*-Aminoheptanophenone of Dog Plasma Samples Obtained for the Protocol Titled "*p*-Aminoheptanophenone (PAHP) (WR269410) Single Dose IV and Oral Pharmacokinetic, Pharmacodynamic, Bioavailability and Metabolism Study in Dogs"	2/7/95 final data	*p*-aminoheptanophenone	dog plasma	189	Pah/P 93-9
Routine Analysis for WR 238, 605 (as f.b.) Monkey Plasma Samples	11/22/94 final data	WR 238,605	monkey plasma	12	WR5/P 94-1

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis for *p*-Amino-heptanophenone Rat Plasma Samples Obtained for the Protocol Titled "p-Aminohept-anophenone (PAHP) (WR 269, 410) Single Dose IV and Oral Pharmacokinetic, Pharmaco-dynamic, Bioavailability and Metabolism Study in Rats"	2/7/95 final data	*p*-aminohept-anophenone	rat plasma	152	Pah/P 94-2
Tentative Title: Routine Analysis for WR 6026 and Metabolites in Plasma and Urine Samples Obtained for the Protocol Titled "Clinical Trial of Oral WR6026•2HCl in Patients with Brazilian Visceral Leishmaniasis due to *L. chagasi*: Initial Dose Range Determine	1/27/97 final data more samples expected	WR 6026 WR 211789 WR 254421	human plasma urine plasma urine plasma urine plasma	 38 37 36 17 24 36 12	WR6/PU 94-3
Tentative Title: Routine Analysis for WR 238605 in Plasma Samples Obtained for the Protocol Titled "Evalua-tion of WR 238605 as a Prophy-lactic Agent against Induced P. falciparum Malaria Infection in Healthy Non-immune Su-bjects: A Dose Ranging Study"	11/21/94 final data	WR 238,605	human plasma blood	 28 28	WR5/PB 94-4
Blind sample results to be added to SR 13B, Supplement II	10/12/94 final data	WR 238,605	dog plasma	30	WR5/P 94-5
Routine Analysis for Pyridostigmine (Cation) in Plasma Samples for the Protocol Titled "A Study to Evaluate the Safety, Tolerance, Pharmacokinetics and Pharmacodynamics of Pyridostigmine when given in Single and Multiple Doses to Males and Females in Diff	4/3/96 final report	Pyridostigmine	human plasma	2639	Pyr/P 94-6

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis for WR 238605 in Plasma Samples Obtained for the Protocol Titled "A Multiple Dose Safety, Tolerance and Pharmacokinetic Study of WR 238605 when Given to Healthy Male and Female Subjects"	8/27/98 final report	WR 238605	human plasma	709	WR5/P 94-7
Routine Analysis for WR 238605 in Rat Plasma Samples Obtained for the Protocol Titled "Six Month Oral Toxicity Study of WR 238605 Succinate in Rats"	7/24/98 final report	WR 238605	rat plasma	405	WR5/P 95-1
Routine Analysis for WR 238, 605 (f.b.) of Human Plasma and Blood Samples and for Chloroquine and Chloroquine Metabolite of Human Blood Samples Obtained for the Protocol Titled "Evaluation of WR 238, 605 as a Prophylactic Agent against Induced *P. falciparum* Malaria Infection in Healthy Non-Immune Subjects II: A Multiple-Dose Causal vs. Suppressive Study"	9/5/97 draft report	WR 238605 chloroquine monodesethyl chloroquine	human plasma blood blood blood	226 226 67 67	WR5/P 95-2
Routine Analysis for the R and S Isomers of WR 238,605 (f.b.) of Human Plasma Samples Obtained for the Protocol Titled "Evaluation of WR 238605 as a Prophylactic Agent against Induced *P. falciparum* Malaria Infection in Healthy Non-Immune Subjects II: A Multiple-Dose Causal versus Suppressive Study"	9/30/97 draft report	*R* WR 238605 *S* WR 238605	human plasma	226 226	WR5/P 95-2
Routine Analysis for WR 238, 605 in Plasma Samples Obtained for the Protocol Titled "WR 238605 Multiple Drug Interaction Study in Beagle Dogs"	8/26/98 preliminary data	WR 238605 Mefloquine Chloroquine Quinine Doxycycline Halofantrine	human plasma	1084	WR5/P 95-3

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis for Halofantrine and WR 178460 in Plasma Samples Obtained for the Protocol Titled "Halofantrine as Prophylaxis against Malaria: Multiple-Dose Safety, Tolerance and Pharmacokinetics Study"	1/3/97 final data chiral assay in progress	Halofantrine WR 178,460 Halofantrine WR 178,460	human plasma	1060 1060 1060 1060	Hal/P 95-4
Routine Analysis for Halofantrine and WR 178460 in Aotus Monkey Blood Samples	1/23/98 final report	Halofantrine	monkey blood	165	Hal/B 96-1
Tentative Title: Routine Analysis for WR 238605 in Plasma Samples Obtained for the Protocol Titled "Dose-Ranging Study of the Safety and Efficacy of WR 238605 in the Prevention of Relapse of Plasmodium vivax Infection in Thailand"	3/2/98 final data submitted in progress	WR238605 Chloroquine	human plasma blood plasma blood	558 552 558 552	WR5/BP 96-2
Routine Analysis for Gentamicin/Paromomycin (as f.b.) of Human Plasma Samples Obtained for the Protocol Titled "Irritant and Phototoxicity Reactions to the Topical Antileischmanial WR 279,369: A Randomized, Double-Blind Phase I Study"	3/12/98 draft report submitted	Gentamicin Paromomycin	human plasma	36 47	Gnt/P 96-3
Routine Analysis for WR238605 in Dog Plasma Samples for the Protocol Titled "One Year Oral Toxicity Study of WR 238605"	9/26/97 final data submitted	WR238605	dog plasma	224	WR5/P 97-1
Routine Analysis for Mefloquine Chloroquine and Primaquine in Plasma Samples	12/11/97 final data	Mefloquine Chloroquine Primaquine	plasma	14 2 2	MEF/P 97-2

DISCUSSION

Methods of Approach

The general development plan is described with emphasis on the laboratory procedures used. We worked on demonstrating sensitivity, specificity, linearity,

lack of interferences, accuracy, and reproducibility of the analytical method, describing the extent of recovery for the method, and reporting on the stability of compounds of interest in specimens during storage and drug analysis. Validation of sensitive and specific analytical methods follow procedures described in the Analytical Section Procedural Manual, Procedure 2D-3.5, "Procedure for Validation of an Assay Methodology" and earlier versions. Methods are developed such that a single technician could complete at least 15 clinical samples in one day. These methods are to be robust and portable enough to be transported to other laboratories.

All drug standards received from the USAMRDC were logged into our record book and stored as required (protected against light, heat, or moisture). If necessary, they were checked for chemical purity or radio-purity by high pressure liquid chromatography or thin layer chromatography, purified through recrystallization or chromatography, and hydroscopic samples were dried according to USP methods.

Sample Preparation for Assay Development

Spiked samples of biological media are prepared by spiking different amounts of drug from known stock solutions into the biological media. Samples are mixed, then equilibrated for up to one hour at room temperature, unless the compound of interest is unstable, in procedures in which it is especially important for measuring drug concentrations in blood, since drugs may take some time to reach equilibrium with erythrocytes.

Sample Preparation Procedures

Suitable preparation of the biological specimens is essential for the successful application of an analytical technique. The preparation procedure should be as simple as possible, yet allow for the specific measurement of the drug in the presence of numerous biological components. The extent of sample work-up is therefore largely determined by the selectivity and sensitivity of the analytical technique. Interfering endogenous substances must be removed before analysis. A second objective in devising preparation steps for a biological specimen is to protect the analytical apparatus from contamination by proteins and undissolved particles. Biological sample preparation thus varies according to the technical demands of the various analytical instruments utilized. Since the advent of highly selective analytical methods that combine chromatographic separation and detection in one unit [e.g. HPLC], the importance of the second objective has become more critical.

Protein Precipitation

Protein precipitation methods are rapid; they involve mixing the sample with water-miscible organic solvents. Acetonitrile yields a protein precipitate that can be readily centrifuged into a small pellet. Use of protein precipitation alone, without further work-up, is a popular application in HPLC analysis. It is possible, using appropriate measurement devices, such as electrochemical or

fluorescence detectors, to obtain adequate sensitivity so that measurements in the nanogram per milliliter range can be made for drugs using small aliquots of the biological sample. We have used the protein precipitation method of sample preparation extensively in the development of analytical assays, including for antibiotics that are zwitterionic in nature, generally possess very low water-to-oil partition coefficients and, thus, are extremely difficult to extract efficiently. Also, protein precipitation is one method of choice for sample preparation, since a simpler sample preparation procedure reduces the risk of degradation. We use the direct protein precipitation method for our studies whenever possible (as demonstrated in Study Report 6 for mefloquine, Study Report 11 for WR 2721 and Study Report 12 for WR 3689).

Lower limits of quantitation with ultraviolet (UV) detectors are usually at about 50 ng/ml concentrations when the protein precipitation method is used. If UV detection is required, organic solvent extraction and solid phase extraction are more useful methods for preparation of biological samples for subsequent analysis. Extraction also limits column overloading and removes assay interferences.

Solvent Extraction

Three major variables were considered in the design of suitable organic solvent extraction procedures: the polarity of the organic solvent, the pH of the aqueous phase, and the volumes of the organic and aqueous phases (as demonstrated in Study Reports 8 and 9 for physostigmine and its metabolite eseroline in plasma and Study Report 10 for WR 6026). A higher pH is often desirable since many endogenous substances are acidic and will not be extracted at alkaline pH. Consideration of pH is therefore important even when assays are developed for neutral drugs. Lipophilic bases are quite uncommon in body fluids, so it should be relatively easy to analyze many of the lipophilic basic drugs by extracting at high pH (as shown in Study Report 13 for WR 238,605 in plasma and blood and Study Report 14 for mefloquine). However, one solvent partitioning step alone is not always capable of separating bases from acids and neutral compounds. In such cases, multiple extraction steps must be employed.

A sample preparation method combining protein precipitation with acetonitrile and extraction with organic solvent is also a viable option. This method has been successfully used in our halofantrine assay ([2] and Study Report 17).

Commercial prepacked solid phase columns [e.g. Bond Elut™] with different types of packing materials, such as silica, C2, C8, C18 and ion exchange were employed. These columns are very useful for sample purification. Two approaches can be utilized: 1] the column separates desired compound(s) from interferences, or 2] the column retains desired compound(s), undesired endogenous substances are washed away, and the desired compound(s) are eluted with a suitable solvent. For low nanogram or picogram per milliliter concentrations, the method of retaining the desired compound on the column is preferred. This method has been successfully used in our laboratory for charged,

water soluble compounds (pyridostigmine (see Study Report 5 for plasma and Study Report 7 for urine)), or highly nonpolar lipophilic, weakly basic and nonvolatile compounds (WR 6026[3] and halofantrine[2]) in biological fluids. For example WR 6026 and halofantrine are non-polar lipophilic compounds which are retained on C8 columns. Pyridostigmine, a quaternary amine, will not elute with CH_3CN alone. A 2 ml CH_3CN wash after loading the biological sample onto the C8 column eliminates undesired substances. The drug is subsequently eluted with CH_3CN containing SDS and tetramethylammonium chloride (TMA+Cl-) or 1% HCl culminating in a quantitation limit of 2 ng/ml with UV detection.

Specific functional groups in molecules of interest can also be advantageously used to purify biological samples by solid phase extraction. Diol functional groups can adsorb on a boronate column and subsequently be eluted with an acidic solution. This turned out to be our method of choice in the ribavirin and WR 249,992 assay development project (see Study Report 15).

Adsorption losses to glass or other apparatus for the low level lipophilic antimalarial drugs probably explains the inconsistent results reported by many investigators. The significance of this adsorption should be considered, especially when several extraction steps are to be employed. This was demonstrated during our development of the assay for halofantrine (WR 171,669) and its active metabolite, WR 178,460, in which WR 194,965 was used as the internal standard ([2] and Study Report 17). The compounds were adsorbed by the glassware after reconstitution of the extract with organic solvent. In our experience, a true measurement of drug was obtained with the addition of a small amount of surface active agent to the solvent system before delivery onto the HPLC column. Adsorption loss can also occur in the port of delivery.

Detector Selection

The detector is a device that supplies an output in response to the presence of the compound(s) of interest. It is connected to the outlet of the column to monitor the column effluent in real time. The detector can be the most sophisticated and one of the most expensive components of a chromatographic system. Optical detectors, which currently dominate the field for biological samples in HPLC, include UV-visible absorbance detectors and fluorescence detectors. Depending on the measured difference between incidental and transmitted light intensity, these instruments can detect down to 9 to 10 ng of sample if the direct precipitation method is used. Electrochemical (EC) detectors are also used for routine work due to their specificity and/or sensitivity.

UV-Visible Absorbance Detector

Since the analytical methods for this contract required the quantitation of nanogram per milliliter concentrations of drug in biological samples, samples assayed with the UV detector required an extensive extraction work-up. For example, the pyridostigmine plasma assay was capable of quantitating 2 ng/ml

concentrations of pyridostigmine (free base) (see Study Report 5) with UV detection only because of the extensive extraction procedure.

Fluorescence Detector

Fluorescence detection is more selective than UV spectroscopy. However, more structural requirements must be met to produce a high fluorescence yield (Ø) and to allow measurement above a negligible background (i.e., better quantitation limits). Minimum detection limits for the fluorescence detector can extend below the nanogram per milliliter level for favorable samples. (See Study Reports 9, 13, and 17).

Fluorescence intensity can be manipulated both by changes in solvent components and the pH of the solvent system. For example, quinoline is non-fluorescent in hexane but fluoresces in ethanol, while indomethacin shows fluorescence at a pH above 12. Most of the synthetic antimalarial drugs are asymmetrically conjugated, not strongly ionic and, hence, would be expected to fluoresce. Fluorescence detection might therefore be expected to be the method of choice for measuring antimalarial drugs due to the sensitivity, selectivity and lower dependence on instrumental stability (from pressure and temperature changes) of the detector.

Two different light sources at various wavelengths are used in commercial fluorescence detectors. They are the deuterium and the xenon arc lamps. The xenon arc lamp has high intensity and the energy is more evenly distributed at different wavelengths, whereas the deuterium lamp emits at lower energy than the xenon arc and the intensity is drastically diminished at wavelengths above 280 nm.

Since the intensity of emitted fluorescence is dependent upon the intensity of the excitation source, it would appear that the sensitivity of a fluorescence assay can be increased without limit by using the most intense source. Many researchers do not realize that marked differences can be found with different lamp sources in commercial detectors.

8-Amino-quinoline antimalarial drugs, such as WR 6026, WR 238,605 and mefloquine (Study Reports 6, 10, 13, 14, 18, and 19) are highly conjugated and the excitation wavelengths were expected to be high. The xenon arc source equipped with monochronometers to collect both the excitation and emitted energy wavelengths provided us with maximum flexibility in fluorescence detection. With these devices, specific wavelengths for optimum sensitivity and/or selectivity were conveniently selected.

Electrochemical Detector

Electrochemical detectors (EC) are also used in methods of choice for applying liquid chromatography to trace (sub-nanogram) analysis. EC detection can provide the sensitivity and selectivity necessary for practical analytical procedures in a variety of situations. Material eluted from the chromatographic

column acts at an electrode surface under controlled potential conditions and the current which results from the net exchange of electrons is monitored as a function of time. Since the amount of material converted by the electrochemical reaction is proportional to the instantaneous concentration, the current will be directly related to the amount of compound eluted as a function of time. The flow through a thin layer electrochemical cell is ideally suited for LC analysis since it can be easily constructed with a very small dead volume (1 µl) and maintain extreme sensitivity toward electroactive compounds. Several configurations using glassy carbon, carbon paste, or mercury-gold electrodes have been developed. If chromatographic conditions are carefully controlled, EC detection is quite precise and quantitative data can be obtained at the picomole level (total injected amount) for many compounds. In addition to being extremely sensitive, the electrochemical detector is quite specific in that only compounds electroactive at a given potential are detected. A large number of extremely important endogenous compounds, drugs, drug metabolites, food additives and organic pollutants are electroactive and therefore can be studied by EC. It is the method of choice for the detection of catecholamines and their analogs; numerous assay methods using EC detection have been published in the recent literature. We have been successful in using this detector for measuring the morphine analog, nalbuphine in urine. When determining whether or not a particular compound can be successfully analyzed by EC, it is not sufficient to know that the compound can react electrochemically. The type of electrode surface, the nature of the solvent system and relative ease of oxidation or reduction must be carefully considered before one can ascertain whether such an analysis is feasible (see Study Reports 11 and 12 for phosphorothioate assays). Many important compounds have been studied in detail and conditions for analysis have been optimized. In order to assess fully the possibility of developing a new assay, it is desirable to carry out voltametric studies. This is equivalent to measuring an adsorption spectrum prior to using a UV detector.

With detection in the reductive mode, analysis of blood for artesunic acid and dihydroquinghaosu had been successfully carried out in Walter Reed Army Institute of Research.[4]

Phosphorothioates ($R\text{-}SPO_3H_2$) are potential radioprotective drugs investigated by the US Army. Neither UV nor fluorescence detection is suitable for this type of compound unless some other functional group in these molecules can be derivatized. To make matters worse, phosphoro-thioates are readily hydrolyzed to free sulfhydryl compounds in vivo (metabolism) and in vitro (degradation) and possibly further oxidized to disulfides. However, phosphorothioates can be detected by EC with dual mercury/gold electrode detectors connected in series. These can be very useful for the simultaneous determination of thiols and disulfides. Two Hg/Au electrodes are utilized in a series arrangement with reduction of disulfide to thiol at the upstream electrode, followed by conventional thiol detection downstream. The upstream electrode behaves as a novel on-line post column reactor of negligible dead volume. Phosphorothioates, thiols and disulfides are all readily quantitated in this detector and suitable separation is achieved by the HPLC system. It is interesting to recall that disulfide is actually being detected as the corresponding

free thiol. No confusion occurs in measurements, however, because thiols are chromatographically resolved from the disulfide and thus separately detected.

LC/MS/MS

Mass spectrometric detectors are increasingly used in methods of choice for applying liquid chromatography to trace (sub-nanogram) analysis. Our Liquid Chromatographic/Mass Spectrometric/Mass Spectrometric (LC/MS/MS) systems for analysis of biological specimens requires development and validation of procedures with PE Sciex-API III® or Micromass Quattro LC Digital P-2 266I systems that use short liquid chromatography columns (3 or 5 μm particle size, 4.6 X 50 mm), the usual liquid chromatographic mobile phases, and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (Atmospheric Pressure Chemical Ionization) or electrospray positive or negative ionization and mass scanning by MRM (Multiple Reaction Monitoring) analysis.

Solvent System and Column

One of the most important steps in the development of an HPLC assay is selection of a suitable solvent system (mobile phase) and stationary phase. They are both closely related for maximum separation. Practical approaches are discussed in this section.

Reverse-Phase and Bonded Phase Columns

We intended to use reverse-phase systems for the majority of the analytical methods developed for HPLC assay described in this contract, since such bonded phase columns have several advantages for applications involving biological fluids. Reverse phase columns are stable since the stationary phase is chemically bonded to the support and cannot easily be removed or lost during use. Therefore, a pre-column and/or pre-saturation of the two phases is/are not required. Reverse-phase columns have minimal irreversible retention which is compatible with a large variety of solvents; it is often possible to inject an aqueous sample without further treatment. As a result, bonded phase columns (BPC) are especially suited for samples containing components with widely varying K' (column capacity factor). The availability of a wide variety of functional groups on BPC packing allows reverse phase and ion paired chromatography to be carried out in a relatively simple, straight-forward manner.

In reverse-phase liquid chromatography, water is the polar solvent and any less polar, water-miscible solvent can be used in conjunction. Common examples of the second solvent include methanol, acetonitrile and tetra-hydrofuran. The design of a successful LC separation depends on matching the right mobile phase to a given column and sample ion pairing mode.

Aqueous Mobile Phase and Silica Columns

The recent use of an unbonded silica stationary phase and an aqueous mobile phase has been successfully used in our laboratory for the liquid chromatographic separation of lipophilic amines. When C18 bonded phase columns are used, it is often necessary to employ amine mobile phase modifiers to ensure good retention times and peak shapes in the ion-suppression mode. Recent work suggests that unbonded silica gel, with the maximum concentration of surface silanol groups, is a preferable stationary phase for these compounds. Use of unbonded silica as the stationary phase permits the separation of a wide variety of amine compounds with a simple mobile phase containing an organic solvent and an aqueous phosphate buffer at neutral to alkaline pH. The retention volumes are lower and the peaks are more symmetrical when silica, rather than a C18 bonded support, is used as the stationary phase. The method is especially suitable for assays of biological fluids, since endogenous non-ionic neutral lipid compounds and anionic compounds will not be retained on the silica gel column while cationic aliphatic amine drugs will be retained. The interfering substances in biological fluids are eluted at the solvent front, leaving a very clean base line about the drug's retention time. Using a silica column and an aqueous solvent system, we obtained a quantitation limit of 2 ng/ml for plasma samples for pyridostigmine (free base) (see Study Report 05).

Selectivity and Resolution Modification

As a general approach to increasing (column selectivity) and improving resolution, several options are available and can be ranked in order of decreasing promise.

Modification of Mobile Phase

Many different properties of the solvent must be considered, including solvent strength and selectivity. Polar compounds are best separated by a polar solvent system, while non-polar compounds should be separated with a less polar system. Separation may be defined as the ability of the solvent system and column material to retain the compound of interest on the column for a longer period of time than the undesired components. We found that a change from methanol to acetonitrile can sometimes enhance the selectivity of the column. We tend to use acetonitrile as the solvent modifier since it has a lower viscosity and tends to increase the efficiency of the column; it is also characterized by increased miscibility with non-polar samples.

Change of pH and Ionic Strength

Aqueous buffers are commonly employed to suppress ionization of the ionizable sample components in reverse-phase analyses. The pH of the mobile phase is varied and the resulting changes in K' (column capacity) and alpha (column selectivity) are examined.

TABLE 3: DRUGS IN PLASMA ASSAYED WITH A SILICA GEL COLUMN AND AN AQUEOUS MOBILE PHASE

Drug	Detection Limit (ng/ml)	Detection Mode	Sample Preparation	Mobile Phase	Retention Time (min.)
WR 238605	0.8	Fluorescence	Liquid extraction	50% CH_3CN 5 mM $(NH_4)_2HPO_4$ pH = 7.0	6.50
WR 6026	1.0	UV	Liquid extraction	60% CH_3CN 5 mM $(NH_4)_2HPO_4$ pH = 7.0	7.00
Halofantrine	1.0	Fluorescence	Liquid extraction	80% CH_3OH 5 mM $(NH_4)_2HPO_4$ pH = 8.2	7.00
Pyrido-stigmine	1.4	UV	Solid Phase extraction	50% CH_3CN, 0.05% TMAC 5 mM $(NH_4)_2HPO_4$ pH = 7.2	16.4
Mefloquine	8.0	UV	Liquid extraction	80% CH_3OH 5 mM $(NH_4)_2HPO_4$ pH = 7.5	10.1

Strength of buffers or ion-pairing agents can also influence the retention times of many drugs. Most of the antimalarial drugs are highly hydrophobic in nature, hence a high ratio of organic solvent modifier should be required and ion-pair techniques will be involved.

For silica stationary and aqueous mobile phase systems, the interaction between silica and amine is electrostatic and the separation mechanism is similar to an ion-exchange mechanism. Here, the pH of the mobile phase (pH 7-9.5) and pK_a of the amine are very important in determining retention time, while the pH of the mobile phase in bonded phase systems (pH 2-5) is not as critical. Ionic strength is also critical. Thus for silica gel-aqueous mobile phase systems, mobile phase pH and ionic strength are more important to retention time determination than the organic modifier (e.g. CH_3CN, CH_3OH), which is the critical determinant in bonded gel - reverse-phase systems.

Change of Stationary Phase

A change of stationary phase is less convenient than a change in mobile phase composition and is less commonly used. Further adjustment of the mobile phase

composition is usually required when a new column packing or stationary phase is used to optimize of solvent strength and K' values.

Most aromatic antimalarial drugs are very non-polar. It is reasonable to expect that the retention time will be shorter on a more polar C8 column (tend not to retain) than a non-polar C18 column. This was found to be true during the development of our first WR 171,669 (halofantrine) assay. The retention time for this compound is reduced by 1/3 by changing from a C18 column to a C8 column when the same mobile phase is used.

Temperature Change

The fourth technique for varying K' values is to increase (or decrease) the temperature. Since an increase in temperature normally reduces all sample K' values, it is usually necessary to decrease solvent strength to compensate for this effect. A change in temperature usually has little effect on sample K' values in liquid-liquid chromatography, but it is important in ion exchange and ion-pair chromatography. For this reason, a change in temperature for improvement of K' in ion-exchange and ion-pair chromatography is generally more promising than a change in stationary phase.

Complexation

A final means of changing K' values, sometimes dramatically, is through chemical complexation. A well known example is the use of metal ions (e.g. $Ag^+NO_3^-$) in the solvent system to separate various olefinic compounds. The complexation of olefin and metal ion causes dramatic changes in retention time and selectivity.[5] This technique is probably applicable for some antimalarial drugs.

For the most part, we intended to use C8 columns and/or ion-pair techniques to develop assay methodologies. However, silica gel column - aqueous mobile phase systems are our general method of choice for amines. Since measurement concentrations of 5 to 20 ng/ml are required, we expected to use 5 μm particle size columns for separation of drugs.

Derivatization

Derivatization is an important adjunct to HPLC assays. The choice of derivatization procedure is dependent upon the type of detector that is used. We are actively involved in pre-column and post-column derivatization as well as in structure modification studies to increase detection sensitivity. A wealth of information on potentially useful derivatives is available from the disciplines of qualitative organic analysis[6] and protective group synthesis.[7] In choosing a derivative for HPLC, ideally the reaction should be specific, quantitative, free from side reactions, complete in a relatively short time, and done under mild conditions. This kind of information is not readily available in the literature, and therefore, derivatization studies can be a rather time consuming venture. The design or choice of a derivatizing agent is critical.

Post column derivatization or degradation is also an excellent way to increase sensitivity of an assay. The technique of post column hydrolysis at alkaline pH and post column oxidation reactions with potassium permanganate or potassium periodate can be applied to assays that employ fluorescence detection. Post-column photo-irradiation is another way to increase sensitivity. First, the drug of interest is separated from other components of the sample by HPLC. Then, the sensitivity is enhanced by photo-irradiation which may rearrange the chromophore or otherwise break bonds to form a fluorescent species

Assay Validation

Validation of the methods were performed using biological fluids obtained from same species, when possible. This process indicates sample stability, method precision, accuracy and selectivity, and the feasible sample concentration range for use in pharmacokinetic or bioavailability studies. Validation procedures are part of our standard operating procedures (SOP) which are written in accordance with our program to meet Good Laboratory Practice (GLP) regulations. The procedures are described in the Analytical Section Procedural Manual, Procedure 2D-3.2 "Procedure for Validation of an Assay Methodology." This section summarizes Procedure 2D-3.2.

Specificity

The specificity should be evidenced by showing with chromatograms that: Test compounds are separated from major metabolites (if metabolite standard is available); Test compounds are separated from co-administered drugs (if any); At least three different sources of biological fluid should be free of possible interference by endogenous peaks at the retention times of the test compounds.

All assay methods developed required use of an internal standard. Analogs of the compounds under study or chemicals with similar functional groups were preferred as internal standards. The internal standard must elute at a different time than the drug of interest, yet separate from endogenous substances in the biological sample. In addition, it should have similar extraction or partition properties as the drug of interest during the sample preparation process.

Linearity

Linearity is demonstrated by acceptable spiked vs. calculated concentrations (or vs. peak response ratios), y-intercept, and coefficient of determination (r^2) values for the standard curve.

Calibration curves were constructed from the peak height (or peak area) ratio of drug to internal standard versus spiked concentration of drug by linear regression (unweighted or weighted method).

In the weighted least squares linear regression method, weights (w) = $1/y_i$, the intercept, b, is defined by:

$$b = \frac{\left(\left(\sum w_i x_i^2\right)\left(\sum w_i y_i\right)\right) - \left(\left(\sum w_i x_i\right)\left(\sum w_i x_i y_i\right)\right)}{\left(\left(\sum w_i\right)\left(\sum w_i x_i^2\right)\right) - \left(\sum w_i x_i\right)^2}$$

and the slope, m, is defined by:

$$m = \frac{\left(\left(\sum w_i\right)\left(\sum w_i x_i y_i\right)\right) - \left(\left(\sum w_i x_i\right)\left(\sum w_i y_i\right)\right)}{\left(\left(\sum w_i\right)\left(\sum w_i x_i^2\right)\right) - \left(\sum w_i x_i\right)^2}$$

Two standard curves may be calculated from the same set of standard curve calibrators (unless the weighted linear regression method is used). The low range curve is calculated from low concentration standard curve points and is used to derive concentrations from samples with peak response ratios at or below the calculated peak response ratio of the highest standard curve point used in the low range curve. The high range curve is calculated from all standard curve points and is used to derive concentrations from samples with peak response ratios above the calculated peak response ratio of the highest standard curve point used in the low range curve.

Standard curve results are reported in a table containing spiked concentrations, peak response ratios, calculated concentrations, slope(s), intercept(s) and r^2 value(s) of a typical standard curve that was used in the method validation and in a table containing all slopes, intercepts and r^2 values of standard curves run in "intraday" and "interday" studies.

Lower Limit of Quantitation

The lower limit of quantitation is defined as the lowest standard curve concentration which can be reasonably, accurately, and precisely quantitated. Six samples spiked to the lowest standard curve concentration and a standard curve are prepared. The samples are run together within one day (or one run). The 6 lowest point of the standard curve sample concentrations, and their mean, S.D., C.V. (percent) and deviation (percent) are calculated. These data are used as the quantitation limit intraday result.

The 6 calculated lowest point of the standard curve concentrations that were obtained in the interday precision study and their means, S.D.s, C.V. percents and deviation percents are used as the quantitation limit interday result.

Recovery

It is important to check the recovery of compounds of interest during the assay in order to assess the uniformity of recovery during the assay or whether or not a better recovery can be obtained. Radio-labeled drugs, when necessary, were added to the sample and either the direct precipitation, solid phase purification or the extraction procedure was utilized to evaluate recovery. If labeled compounds were not available, a recovery study similar to those for WR

6026,[3] halofantrine and its metabolite, WR 178,460,[2] and pyridostigmine[8] were carried out. In brief, the recoveries of these drugs from plasma or whole blood were determined by comparison of the drug-to-internal standard peak height ratios of blood or plasma versus water samples spiked with the drug. In each case, the internal standard was added after sample was eluted from the solid phase column, extraction from organic solvent, or direct precipitation with CH_3CN to insure that the internal standard did not bind to the blood or plasma or to the cartridge during the preparation.

Precision

Precision is expressed as the standard deviation (S) of the assayed concentration where Xi are the repeated concentration measurements of an individual sample and $\overline{x}$ is the mean concentration.

$$S = \left(\frac{\sum_{i=1}^{N} (Xi - \overline{x})2}{(N-1)} \right)^{\frac{1}{2}}$$

The coefficient of variation (C.V.) was used for determination of the precision. The sample number was 6 for intraday and 12 for interday precision. The bias of an assay method is determined by comparing biological sample results with spiked values. The significance of the bias is established by setting a confidence limit.

$$\text{Percent C.V.} = \frac{S \bullet 100}{\overline{x}}, \quad (N \geq 6)$$

If needed, the assay results were compared to those obtained with an assay of proven reliability and specificity. For example, the Pearson correlation coefficient (r) can be used. Maximum r value indicates exact correlation between the two variables and r = 0 indicates complete independence.

$$r = \frac{(Si - \overline{s})(Yi - \overline{y})}{n \bullet Sx \bullet Sy}$$

The within-run precision was determined by measuring the amount of drug in a number of biological samples, in duplicate. The duplicate mean results are used to calculate the standard deviation. The between-run precision is measured on separate days with replicate samples at low, intermediate and high drug concentrations. From these three sets of replicate samples, the between-run standard deviation is calculated for each drug concentration.

Accuracy

Accuracy was determined by assaying a series of blind samples prepared according to the project director of DAMD. Estimates of the accuracy of the method over the standard curve working range were also determined in the

precision analysis by the analysis of replicate spiked samples for intraday (n = 6) and interday (n = 12) precision. Results were expressed as relative error (RE) with respect to the spiked concentration.

Stability

Stability studies of a drug in biological media serve to establish the procedure for proper storage of the samples and furnishes information to clinical researchers on how best to handle these occasionally labile samples. We have a great deal of experience in planning and executing the required stability studies. In methods developed for analytical and clinical studies, drug stability may play a particularly important role.

Known amounts of sample in different biological media are measured at various times after preparation and assayed for the drug, in duplicate. Variables, including light exposure, storage conditions (container type) and pH of the biological samples, are evaluated if necessary.

Since clinical samples are often repeatedly assayed and samples are thawed and refrozen, it is necessary to check for any instability of samples during these processes. Practically, this study can be done by using two concentration samples (High and Low). The same volume of biological fluid used to prepare standard curve samples is aliquoted to the appropriate number of tubes. Samples (in duplicate) are thawed and refrozen (a cycle) for 5 cycles. Samples are repeatedly thawed and refrozen according to the following table. Samples are thawed as if for sample preparation to room temperature and are left to stand at room temperature for 1 hour.

Cycle	Keep these samples in freezer
1	a
2	a, b
3	a, b, c
4	a, b, c, d
5	a, b, c, d, e

Following Cycle 5, all of the samples are thawed to room temperature and assayed with a standard curve. Test sample concentrations are calculated and reported in a table for each concentration (n=2) of mean concentrations (n=2) at each test point (n=5).

System (processed sample) stability: Stability of drug and internal standard in biological samples prepared for analysis as described above under "Sample Preparation" were demonstrated by assay of sets of control samples with concentrations to cover the standard curve range. Assay results were obtained for prepared samples that were left standing at room temperature for various times after preparation.

Refrigerated (processed sample, refrigerated) stability: Assay results were obtained for prepared samples that were left standing at 4°C for various times after preparation.

Long term stability: Stability of a drug in a biological specimen stored at -20°C and/or -70°C was demonstrated by assay of sets of control samples prepared as described under "Sample Preparation" at concentrations to cover the standard curve range. Long term stability samples were kept at -70°C or -20°C until prepared and analyzed.

Bench top (unprocessed sample) stability: Plasma samples were left to stand at room temperature for various times after generation, then were kept at -20°C and/or -70°C until prepared and analyzed.

Purity of Standard Chemicals

The standard chemicals used in a study are USP™ reference standards (if available) or pure chemicals provided by the sponsor, unless otherwise instructed.

A chemical purchased from a general chemical company is not used as a standard, except in unusual circumstances and when its purity can be verified against a USP™ reference standard or the sponsor's standard verified by a certificate of analysis. The internal standard is not under this restriction.

USP™ reference standards can be regarded as 100% pure (unless specified), and no purity correction factor for concentration calculations is necessary. However, a sponsor's standard chemical must be regarded as possibly impure and a correction factor should be considered.

When verifying non USP™ reference standard chemical purity, the working standard solution is run under the method used for assaying biological samples. Each solution is injected 3 times, and two standard solutions are prepared for each standard chemical.

A copy of the supplier's certification sheet is saved with the method validation files.

Routine Assay Procedure

The following are the steps carried out when samples arrive for routine analysis. Sample arrival is recorded in the sample log-in book and on the log-in sheet, which includes the name of the shipper, arrival date, number of samples, sample storage location, and sample condition. An analytical procedure (AP) is normally completed prior to routine analysis. In this AP, the method description is condensed to about 3-10 pages that contain information regarding instrumentation, assay conditions, source of chemicals, preparation of stock solutions, sample preparation and representative chromatograms.

For routine sample analysis, standard curve, blank and control samples are also analyzed. Sets of equipment consisting of a pump, detector, column, integrator and autosampler or the LC/MS/MS system were set up for routine assay. Each system is tested by assigning personnel to run a series of controls; the performance of equipment and technical personnel are validated before routine sample assay.

Carry over testing is performed for each run by assay of a blank sample (not spiked with internal standard or with drug standard) immediately following the assay of a high concentration control.

To monitor variation during the course of assay of a sufficient quantity of samples, a series of controls are prepared beforehand and stored in the freezer. Control samples are run together with standards and routine assay samples. Each set of controls normally includes three different concentrations within the range of the standard curve. For every group, treatment, or up to 20 routine assay samples, a set of controls (e.g. low, medium and high) is included before and after the set to validate the results. Since the concentrations of the controls are known, it is possible to judge whether the routine assay samples must be repeated on the basis of the results obtained for the controls.

Assay samples are prepared by spiking known volumes of biological sample with a known amount (constant over all samples) of internal standard (IS). Standard curve samples are generated by spiking interference free biological samples with known amounts of standard compound and IS. These standard curve and assay samples are prepared according to the analytical procedure, then injected onto an LC column for separation and subsequent detection. The peak response ratio of standard compound to IS is calculated for each sample from the measured peak response obtained by HPLC or LC/MS/MS. Finally, spiked concentrations and standard compound to IS peak response ratios of the standard curve samples are fit by weighted or non weighted least squares linear regression to the equation for the best straight line ($y = mx + b$, where y = peak response ratio and x = standard compound concentration), and standard compound concentrations in assay samples are calculated by this equation from the standard compound to IS peak response ratios obtained by HPLC or LC/MS/MS.

Assay findings are then sent in a report with a complete assay methodology including detailed methods, statistical evaluation of methods, routine assay sample results, results from the control samples, and one representative set of calibration chromatograms. Results can be sent by disc or through a modem for pharmacokinetic evaluation.

Experimental methods

The goals of the research under contract DAMD17-97-C-7058 are 1) to develop and validate methods to assay for drug substances in biological fluids for pharmacokinetic, bioavailability, drug metabolism and drug monitoring studies, and 2) to use these methods to perform routine analyses of biological

specimens to support pharmacokinetic and bioavailability studies as part of preclinical and clinical investigations undertaken for the purpose of new drug development.

Method Development and/or Validation Results

The following section describes the status of specific methods developed and validated or currently being developed and/or validated during the contract.

TABLE 4: CURRENT VALIDATION STUDIES

Report No.	Report Date	Report Title	Test System	Test Article	Lower Limit of Quantitation
18	Status Report 7/31/91 Final report in preparation	Quantitation of WR 6026 and WR 211,789 (WR 6026 Metabolite) in Plasma and Blood by HPLC with a Silica Gel Column and an Aqueous Mobile Phase	Plasma blood	WR 6026 WR 211789 WR 6026 WR 211789	0.980 ng/ml 1.21 ng/ml 0.980 ng/ml 1.21 ng/ml
19	1/14/92 in revision	Quantitation of Mefloquine in Human Blood By HPLC, Extraction Method	human blood	mefloquine WR 160972	7.36 ng/ml ng/ml
21	Draft report in preparation	Tentative title: Quantitation of *p*-Aminoheptanophenone, *p*-Aminooctanophenone, and *p*-Aminopropiophenone in Dog Plasma by HPLC	dog plasma	PAHP PAPP PAOP	4.08 ng/ml 4.04 ng/ml 4.16 ng/ml
22	Draft 7/18/94 in revision	Quantitation of WR 6026, WR 211,789, and WR 254,421 (as Free Bases) in Human Urine By HPLC	human urine	WR 6026 WR 211,789 WR 254,421	5.17 ng/ml 5.09 ng/ml 45.4 ng/ml
26	12/12/96 final report, amendment in preparation	Quantitation of WR 242511 (as Free Base) in Human and Dog Plasma By HPLC with a Silica Gel Column and an Aqueous Mobile Phase	human plasma dog plasma	WR 242511 WR 242511	4.00 ng/ml 4.00 ng/ml
28	Draft in preparation	Tentative title: Quantitation of R&S Isomers of Halofantrine and WR 178,460 in Human Plasma by HPLC	human plasma	Halofantrine WR 178,460	ng/ml ng/ml
29	8/22/97 status report in revision	Validation of a LC/MS/MS Method for the Determination of Chloroquine and Monodesethylchloroquine in Human Blood Samples	human blood	Chloroquine (C) MonodesethylC	20 ng/ml 20 ng/ml
29B		Validation of a LC/MS/MS Method for the Determination of Chloroquine and Monodes-ethylchloroquine & Dides-ethylchloroquine in Human Blood & PlasmaSamples	human blood human plasma	Chloroquine (C) MonodesethylC DidesethylC Chloroquine (C) MonodesethylC DidesethylC	ng/ml ng/ml ng/ml ng/ml ng/ml ng/ml

TABLE 4: CURRENT VALIDATION STUDIES

Report No.	Report Date	Report Title	Test System	Test Article	Lower Limit of Quantitation
30	In validation	Tentative title: Quantitation of WR 243251 in Human Plasma by LC/MS/MS	human plasma	WR 243251	1 to 5 ng/ml
31 part 1	Report dated May 10, 1999	Validation of LC/MS/MS Method for the Determination of Chloroquine (& its metabolites), Quinine, Doxycycline, Halofantrine (& its metabolite), Mefloquine, and WR 238,605 in Dog Plasma Samples, Part I: WR 238,605 and Mefloquine	dog plasma	WR 238605 Mefloquine	4.00 ng/ml 5.00 ng/ml
31 part 2	Report dated May 13, 1999	Part II: Chloroquine and Quinine	dog plasma	Chloroquine (C) MonodesethylC DidesethylC Quinine	4.00 ng/ml 4.00 ng/ml 4.00 ng/ml 10.0 ng/ml
31 part 3	March 24, 1999	Part III: Doxycycline	dog plasma	Doxycycline	50.0 ng/ml
31 part 4	April 22, 1999	Part IV: Halofantrine (and its metabolite) and WR 238,605	dog plasma	Halofantrine WR 178,460 WR 238,605	2.00 ng/ml 2.00 ng/ml 2.00 ng/ml
32	Draft in preparation	Tentative title: Quantitation of WR 238,605 in Human Plasma and Small Volume in Human Blood by LC/MS/MS and	human plasma blood	WR 238605 WR 238605	ng/ml ng/ml
33	In validation	Tentative title: Quantitation of Halofantrine and WR 178,460 in Human Plasma by LC/MS/MS	human plasma	Halofantrine WR 178,460	ng/ml ng/ml
34	In development	Tentative title: Quantitation of WR 254421 in Human Plasma by LC/MS/MS	human plasma	WR 254421	ng/ml
35	In development	Tentative title: Validation of LC/MS/MS Method for the Determination of Artelinic Acid in Rat and Dog & Human Plasma	rat plasma dog plasma	Artelinic Acid Artelinic Acid	ng/ml ng/ml

Study Report 18: WR 6026 and Metabolite in Human Plasma and Blood

Study Characteristics: Study Report 18	
Test Article:	WR 6026, WR 211,789
Test System:	human plasma and blood
Internal Standard:	chlorpheniramine
Sample Assay Volume:	0.5 ml
Sample Cleanup:	methyl *t*-butyl ether extraction
Analytical System	
Detector:	UV at 263 nm
Column Type:	silica
Column Size:	4.6x250 mm, 5µ particle size
Mobile Phase:	acetonitrile/water (3:2, v/v) final concentration of 5 mM $(NH_4)_2HPO_4$ at pH 8.8
Validation Results: WR 6026 in human plasma	
Quantitation Limit:	0.980 ng/ml
Standard curve range:	0.980-98.0 ng/ml
Interday Precision	
Concentration Range:	2.06-77.3 ng/ml
CV Range:	3.05-6.82%
Intraday Precision	
Concentration Range:	2.06-77.3 ng/ml
CV Range:	3.22-9.39%
Blind Sample Assay	see Appendix A, DAMD17-92-C-2028 Midterm Report
Mean Recovery:	74.5%
Stable Plasma Storage:	-20°C for 3 months
Validation Results: WR 211,789 in human plasma	
Quantitation Limit:	1.21 ng/ml
Standard curve range:	1.21-121 ng/ml
Interday Precision	
Concentration Range:	2.14-80.1 ng/ml
CV Range:	5.19-8.98%
Intraday Precision	
Concentration Range:	2.14-80.1 ng/ml
CV Range:	4.42-7.86%
Blind Sample Assay	see Appendix A, DAMD17-92-C-2028 Midterm Report
Mean Recovery:	93.8%

Validation Results: WR 6026 in human blood

Quantitation Limit:	0.980 ng/ml
Standard curve range:	0.980-98.0 ng/ml
Interday Precision	
Concentration Range:	1.96-78.4 ng/ml
CV Range:	1.56-6.38%
Intraday Precision	
Concentration Range:	1.96-78.4 ng/ml
CV Range:	2.31-5.36%
Stable Plasma Storage:	-20°C for 1 month -70°C for 3 months

Validation Results: WR 211,789 in human blood

Quantitation Limit:	1.21 ng/ml
Standard curve range:	1.21-121 ng/ml
Interday Precision	
Concentration Range:	2.40-96.0 ng/ml
CV Range:	1.74-5.12%
Intraday Precision	
Concentration Range:	2.40-96.0 ng/ml
CV Range:	1.76-4.85%

Study Description: WR 6026 and Metabolite in Human Plasma and Blood (the methodology was presented in DAMD17-92-C-2028 mid-term report)

Sets of blind plasma and blood samples, prepared April 1, 1993, were received. Blind plasma sample results were enclosed with Quarterly Report 8. Upon analysis of blood samples, results will be forwarded to the COR. Acceptable results will be incorporated into Study Report 18, "Quantitation of WR 6026 and WR 211,789 (as Free Bases) in Plasma and Blood by High-Performance Liquid Chromatography." The test of stability is in progress.
Procedures Required to Complete Validation

The following list details changes that were instituted for plasma sample analysis, but that have not been tested for validation of the blood sample analytical method.

1. Following addition of 5 ml of methyl-*t*-butyl ether, vortex [not rotate] samples for 1 [not 15] min.
2. Adjust the mobile phase pH to 8.8 [not 7.0].
3. Stock and working solutions were stored at -20°C [not 4°C].

The following list details validation tests that have not been done.

1. Stability of WR 211,789 (free base) at -80°C and -20°C in blood and plasma.
2. Recovery of WR 6026 and WR 211,789 from blood.

3. Precision of WR 6026 and WR 211,789 (as free bases) in blood with mobile phase pH = 8.8, storage of stock and working solutions at -20°C, and vortexing extraction samples for 1 min.

4. Accuracy for WR 6026 and WR 211,789 (as free bases) in plasma and blood on blind spiked samples prepared by the Walter Reed Army Institute of Research.

5. Interference: To determine whether known compounds would interfere with detection of WR 6026 or WR 211,789 (as free bases), the retention times relative to CPA in mobile phase of several WR 6026 (free base) analogs could include WR 225,742 and WR 254,421 (free base).

Study Description

WR 6026 (dihydrochloride) (6-methoxy-8-(6-diethyl amino hexyl amino) lepidine dihydrochloride) (see figure below), is a very effective antileishmanial drug in hamsters infected with *Leishmania donovani* .[9]

Because antimony compounds are not always effective and the other drugs in use have toxic effects,[10,11] alternative therapies are needed. Since WR 6026 (dihydrochloride) is a likely candidate and since WR 6026 (dihydro-chloride) is scheduled for clinical testing in the near future, it is extremely important to develop an analytical method capable of measuring concentra-tions of WR 6026 (free base) at nanogram per milliliter concentrations in biological samples.

This report describes an assay developed to determine the concentrations of WR 6026 and of its mono dealkylated metabolite, WR 211,789, (as free bases) in blood and plasma. This new assay provides significant improvements over capabilities of earlier assays with increased sensitivity for the detection of WR 6026 (free base)[12]and inclusion of WR 211,789 (free base) in the methodology (Study Report 10).

Plasma samples (0.5 ml transferred with a plastic tipped pipetter to silanized culture tubes (see SOP #3-11 for silanization procedure)) were vortexed with 100 µl of a 1.00 µg/ml chlorpheniramine maleate internal standard working solution and 100 µl of a 1 N NaOH solution for 10 s. Next, 5 ml of methyl-*t*-butyl ether was added and samples were vortexed for 1 min, then centrifuged for 10 min at 3000 *g*. Then, for each sample, the aqueous layer was frozen in a dry ice/methanol bath and the organic layer were decanted into a new silanized culture tube. Finally, the sample's organic layer was evaporated to dryness under prepurified nitrogen, reconstituted in 200 µl of mobile phase, vortexed for 1 min, transferred to silanized WISP inserts, and injected onto the HPLC column.

Blood samples (0.5 ml transferred with a plastic tipped pipetter to silanized culture tubes) were vortexed for 1 min with 0.5 ml of nanopure water, and the mixtures were sonicated for 10 min. Then, these samples were prepared like plasma samples beginning with addition of 100 µl of the internal standard working solution.

No degradation of WR 6026 (free base) in plasma frozen at -20°C or blood frozen at -80°C was seen for the duration of the stability study. However, noticeable degradation of WR 6026 (free base) in blood frozen at -20°C was observed by the third month at all concentrations.

Two standard curves for each assay were constructed from the chromatographic data; a low range curve from the 0 to 14.7 ng/ml for WR 6026 and 0 to 18.1 ng/ml for WR 211,789 standard curve samples and a high range curve from the 0 to 98.0 ng/ml for WR 6026 and 0 to 121 ng/ml (i.e. all) standard curve samples in order to obtain more accurate determinations of low level WR 6026 and WR 211,789 (free base) concentrations. The low range standard curve was used to calculate drug or metabolite concentrations for assayed samples when the peak height ratio of the sample was less than or equal to the calculated peak height ratio at the highest concentration of the low range curve (as calculated from the low range curve). The high range curve was used to calculate results for samples with peak height ratios greater than the calculated peak height ratio at the highest concentration of the low range curve (as calculated from the low range curve).

Typical plasma and blood chromatograms show WR 6026 (free base), WR 211,789 (free base) and internal standard, chlorpheniramine, peaks that are baseline separated and separated from other components of the sample.

A linear relationships was demonstrated between the WR 6026 and WR 211,789 (free base) spiked concentrations to the WR 6026 and WR 211,789 (free base) to internal standard peak height ratios for the plasma and blood assays. Linear regression analysis of concentration versus the peak height ratio gave coefficients of determination (r^2) of 0.989 or better for these typical standard curves. The linear range of the standard curves covered WR 6026 (free base) concentrations in plasma and blood in the range 0.980 to 98.0 ng/ml and WR 211,789 (free base) concentrations in plasma and blood in the range 1.21 to 121 ng/ml. The reversed-phase system (alkyl bonded silica gel with an aqueous mobile phase) is the most widely used HPLC technique in assays for drugs in biological fluids. In this kind of a system, the retention mechanism depends mainly on the lipophilic character of substances to be analyzed. Such a mechanism also retains considerable amounts of other lipophilic substances, thereby interfering with the drug peak. On the other hand, in a system consisting of a bare silica gel and an aqueous mobile phase, the retention mechanism results mainly from ion exchange[13] and only partially from lipophilic interactions. Thus, endogenous non-ionic neutral lipid compounds and anionic compounds will not be retained on the silica gel column; only the cationic (e.g. ammonium) ions will be retained. The interfering substances in biological fluids elute at the solvent front, leaving a very clean baseline around the retention time of the drug.

Validation trials in our laboratory for an earlier study (Study Report 10) were undertaken to include in the WR 6026 (free base) assay the capability to measure WR 211,789 (free base), a mono dealkylated metabolite of WR 6026 (free base), concentrations in biological samples. Large variations between spiked and

recovered concentrations were observed in that study. Although WR 211,789 has been detected in a rat microsomal preparation,[14] it has not been detected in plasma in human studies, perhaps because the detection limit of the assay used was only 10 ng/ml.[19] WR 211,789 plasma standard curves in the trials were of higher quality than blood standard curves. The current report describes an adaptation of the WR 6026 (free base) methodology or a modification of the methodology presented in the earlier report (Study Report 10), in which a 5 to 10 fold increase in sensitivity has been gained that makes detection of WR 211,789 (free base) in human plasma possible at higher WR 6026 (dihydrochloride) doses.

In addition, compared to an even earlier methodology,[19] the WR 6026 (free base) HPLC method presented here offers increased sensitivity and extends the range of biological fluids that can be assayed. The earlier method measured WR 6026 (free base) in plasma cleaned by protein precipitation (with acetonitrile) and column elution (from a C2 extraction column), had a 6.44 ng/ml WR 6026 (free base) detection limit, used WR 223,658 as an internal standard, required a C8 bonded silica gel HPLC column, used a 60:40 (v/v) acetonitrile/water mobile phase at pH 5.5 with 0.2% final concentrations of SDS and glacial acetic acid, and measured WR 211,789 (free base) with a minimum detection limit of 8 ng/ml. The newer method measures WR 6026 (free base) in plasma and blood cleaned by extraction with 99:1 (v/v) pentane/acetonitrile, has a 0.980 ng/ml WR 6026 (free base) detection limit, uses chlorpheniramine maleate as an internal standard, requires an unbonded silica gel HPLC column, uses a 70:30 (v/v) acetonitrile/water mobile phase at pH 7.0 with 5 mM final concentration of dibasic ammonium phosphate, but could not measure WR 211,789 (free base) with a minimum detection limit much better than 8 ng/ml. The current modified method measures WR 6026 and WR 211,789 (free base) in plasma and blood cleaned by extraction with methyl-*t*-butyl ether, has 0.980 ng/ml WR 6026 and 1.21 ng/ml WR 211,789 (as free bases) detection limits, uses chlorpheniramine maleate as an internal standard, requires an unbonded silica gel HPLC column, uses a 60:40 (v/v) acetonitrile/water mobile phase at pH 8.8 with 5 mM final concentration of dibasic ammonium phosphate.

HPLC assays for basic amine drugs in biological samples that make use of a silica gel column and an aqueous mobile phase have been operated in this laboratory for over 5 years.[15,16,17] In the WR 6026 (free base) HPLC method presented here, the use of an unbonded silica gel column, an aqueous mobile phase, and UV detection at 263 nm yields satisfactory results for the determination of WR 6026 and WR 211,789 (as free bases) in (0.5 ml) plasma and blood samples. The method is simple in that a single extraction step and evaporation of solvent prior to injection are required. Recovery of WR 6026 (free base) averaged 74.5%, while recovery of WR 211,789 (free base) averaged 93.8% from plasma. The minimum quantitation limits of the assay were 0.980 ng/ml for WR 6026 (free base) and 1.21 ng/ml for WR 211,789 (free base) for blood and plasma. The coefficients of variation of the inter- and intraday assay precision analyses were less than 10% at all concentrations. The method is simple, precise, more sensitive, and includes the capability of quantitating WR 211,789 (free base) as well as the parent drug compared to earlier methods.

Study Report 19: Mefloquine in Human Blood

Study Characteristics: Study Report 19

Test Article:	Mefloquine
Test System:	human blood
Internal Standard:	chlorpheniramine
Sample Assay Volume:	0.5 ml
Sample Cleanup:	pentane/methylene chloride (7:3, v/v) extraction

Analytical System

Detector:	UV at 280 nm
Column Type:	silica
Column Size:	4.6x250 mm, 5µ particle size
Mobile Phase:	methanol/water (4:1, v/v) final concentration of 5 mM $(NH_4)_2HPO_4$ at pH 7.5

Validation Results: Mefloquine in blood

Quantitation Limit:	7.36 ng/ml
Standard curve range:	7.36-2210 ng/ml
Interday Precision	
Concentration Range:	14.7-1472 ng/ml
CV Range:	3.94-8.41%
Intraday Precision	
Concentration Range:	14.7-1472 ng/ml
CV Range:	2.74-10.9%
Blind Sample Assay	
Concentration Range:	11.52-1536 ng/ml
Bias Range:	-12.6 to +7.20%
Mean Recovery:	91.5%
Stable Blood Storage:	-20°C for 4 months

Study Description: Mefloquine in Human Blood (the methodology was presented in DAMD17-92-C-2028 mid-term report)

Mefloquine (hydrochloride), (WR 142,490: erythro-a-(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol hydrochloride), is an alternative curative agent for the treatment of falciparum malaria.[18,19] Mefloquine (hydrochloride) has also been shown to prophylactically suppress mosquito induced infections by *Plasmodium vivax* and *P. falciparum* in human volunteers.[20,21] Published plasma and/or blood analytical methods employ gas-liquid chromatography (GLC)[22,23,24] thin layer chromatography (TLC),[25] gas chromatography-mass spectrometry (GC-MS)[26] or high performance liquid chromatography (HPLC) (Study Reports 6 and 14).[27,28,29,30,31,32,33] The GLC

methods require derivitization, and sample volume in the method described by Nakagawa, *et al.* uses 5 ml samples. The TLC method has no internal standard and is insufficiently sensitive. The GC-MS method requires derivitization and the increased expense of mass spectrometry.

Many HPLC have been reported. The method reported by Grindel, *et al.*, required three times extraction from 5 ml plasma samples and, upon solvent evaporation, the residues required overnight storage in a vacuum desiccator. Kapetanovic, *et al.* used a 3 step extraction of 1 ml samples. Our earlier study (Study Report 6) described a protein precipitation method for 0.2 ml plasma samples. Franssen, *et al.*, described a method for plasma and blood analysis for mefloquine and its carboxylic acid metabolite with 50 ng/ml mefloquine and 100 ng/ml metabolite detection limits. Karbwang, *et al.*, described a 50 ng/ml detection limit, 100 ng/ml quantitation limit method for mefloquine in blood and plasma. Coleman, *et al.*, measured mefloquine at 10 ng/ml in liver perfusate. Riviere *et al.*, presented a method with a 20 ng/ml detection limit and 100 ng/ml quantitation limit in plasma. Bergqvist, *et al.*, describe two HPLC methods for determination of mefloquine and its principal metabolite in plasma and blood, the first with 30 ng/ml plasma and 150 ng/ml blood quantitation limits and the second with 75 ng/ml quantitation limits for both compounds.

We reported (Study Report 14) the development of a simple and rapid HPLC assay for mefloquine (free base) that requires 0.5 ml plasma samples and a one step extraction, has an 7.36 ng/ml quantitation limit and produces chromatograms with a cleaner baseline than our previous method. Study Report 19 describes the extension of our plasma method to include analysis of blood samples. Study Report 19 also describes status of steps taken toward extension of the method for determination of the main mefloquine metabolite, WR 160,972 (2,8-bis-(trifluoromethyl)-4-quinoline carboxylic acid).[34]

The blood method was modified from the plasma method described in Study Report 14, "Quantitation of Mefloquine (Free Base) in Plasma by High-Performance Liquid Chromatography, Extraction Method." The blood method primarily differs from the plasma method in sample preparation by:

1. Allowing blood standard curve calibrator samples to equilibrate for 1 hour following spiking with mefloquine working solutions;

2. Addition of 0.5 ml water; and

3. Sonication for 10 min prior to addition of internal standard.

Blood samples for analysis are pipetted (0.5 ml) into screw top tubes. Add 100 µl of a saturated solution of sodium carbonate and vortex the mixture for 1 min. Then, add 100 µl of the internal standard working solution (CPA, 12 µg/ml) and vortex the mixture for 1 min. The sample is extracted with 5 ml of pentane/methylene chloride (7:3, v/v), evaporated to dryness under nitrogen, resuspended in 200 µl of mobile phase and injected (40-80 µl) onto the HPLC column.

An addendum with blind sample results enclosed with Quarterly Report 6 completed Study Report 19 (Status Report, dated January 14, 1992 and titled "Quantitation of Mefloquine (Free Base) in Blood by High-Performance Liquid Chromatography, Extraction Method." Further work on this assay is scheduled to include WR 160,972 method development, but work on this aspect of the project has been assigned a low priority by the COR.

Study Report 21: *p*-Aminoheptanophenone and Metabolites in Dog Plasma and Rat Plasma

Study Characteristics: Study Report 21	
Test Article:	WR 269,410 (*p*-aminoheptanophenone)
Test System:	dog plasma
Internal Standard:	WR 258,948 (*p*-aminooctanophenone)
Sample Assay Volume:	0.5 ml
Sample Cleanup:	methyl *t*-butyl ether extraction
Analytical System	
Detector:	UV at 316 nm
Column Type:	C18 bonded silica
Column Size:	4.6x250 mm, 5µ particle size
Mobile Phase:	acetonitrile/water (1:1, v/v) and 0.15% H_3PO_4
Validation Results:	
Quantitation Limit:	4.08 ng/ml
Standard curve range:	4.08-816 ng/ml
Blind Sample Assay	see Appendix A, DAMD17-92-C-2028 Midterm Report

Study Description: *p*-Aminoheptanophenone and Metabolites in Dog Plasma and Rat Plasma (the analytical procedure was presented in DAMD17-92-C-2028 final report)

Method validation will be reported in Study Report 21 (preparation in progress covering *p*-aminoheptanophenone (PAHP, WR 269,410) *p*-aminooctanophenone (PAOP, WR 258,948) and *p*-aminopropiophenone (PAPP, WR 302)). Results from the analysis of blind spiked (by WRAIR) dog plasma samples were presented in Appendix A of the mid-term report.

Study Report 22: WR 6026 and Metabolites in Human Urine

Study Characteristics: Study Report 22

Test Article:	WR 6026 WR 211,789 WR 254,421
Test System:	human urine
Internal Standard:	verapamil
Sample Assay Volume:	0.5 ml
Sample Cleanup:	methyl *t*-butyl ether extraction

Analytical System

Detector:	UV at 350 nm
Column Type:	silica
Column Size:	4.6x250 mm, 5μ particle size
Mobile Phase:	acetonitrile/0.0075% phosphoric acid (80:20, v/v) at pH 6.9.

Validation Results WR 6026 free base

Quantitation Limit:	5.17 ng/ml
Interday CV:	14.8%
Interday Error:	2.44%
Standard curve range:	2.17-414 ng/ml
Interday Precision	
Concentration Range:	10.4-259 ng/ml
CV Range:	3.90-7.42%
Intraday Precision	
Concentration Range:	10.4-259 ng/ml
CV Range:	3.83-28.4%
Blind Sample Assay	
Concentration Range:	5.20-101.2 ng/ml
Bias Range:	-10.6 to +33.9%
Mean Recovery:	97.2%
Stable Plasma Storage:	-70°C for 4 months
Stable Prepared Sample:	Room temp. for 48 hours

Validation Results WR 211,789 free base

Quantitation Limit:	509 ng/ml
Interday CV:	14.7%
Interday Error:	4.55%
Standard curve range:	5.09-407 ng/ml
Interday Precision	
Concentration Range:	10.2-255 ng/ml
CV Range:	4.07-10.0%
Intraday Precision	
Concentration Range:	10.2-255 ng/ml
CV Range:	5.12-23.3%
Blind Sample Assay	
Concentration Range:	5.20-102.6 ng/ml
Bias Range:	-11.8 to +33.9%
Mean Recovery:	92.8%
Stable Plasma Storage:	-70°C for 4 months
Stable Prepared Sample:	Room temp. for 48 hours

Validation Results WR 254,421 free base

Quantitation Limit:	45.4 ng/ml
Interday CV:	7.51%
Interday Error:	1.60%
Standard curve range:	45.4-3630 ng/ml
Interday Precision	
Concentration Range:	90.8-2270 ng/ml
CV Range:	3.09-5.86%
Intraday Precision	
Concentration Range:	90.8-2270 ng/ml
CV Range:	3.55-10.0%
Blind Sample Assay	
Concentration Range:	50.1-979.4 ng/ml
Bias Range:	-10.7 to +9.63%
Mean Recovery:	94.2%
Stable Plasma Storage:	-70°C for 4 months
Stable Prepared Sample:	Room temp. for 48 hours

Study Description: WR 6026 and Metabolites in Human Urine (the methodology was presented in DAMD17-92-C-2028 final report)

Study Report 22 "Quantitation of WR 6026, WR 211,789 and WR 254,421 (as Free Bases) in Human Urine by High Performance Liquid Chromatography,"

was submitted for review July 18, 1994. This method is a modified version of the plasma method.

WR 6026 (dihydrochloride) (6-methoxy-8-(6-diethyl amino hexyl amino) lepidine dihydrochloride) (Figure 1), is a very effective antileishmanial drug in hamsters infected with *Leishmania donovani* .[16] Because antimony compounds are not always effective and the other drugs in use have toxic effects,[17,18] alternative therapies are needed. Since WR 6026 (dihydrochloride) is a likely candidate and since WR 6026 (dihydro-chloride) is scheduled for clinical testing, it is extremely important to develop an analytical method capable of measuring concentrations of WR 6026 (free base) at nanogram per milliliter concentrations in biological samples. This report describes an assay developed to determine the concentrations (as free bases) of WR 6026 and of its metabolites, WR 211,789 (6-methoxy-8-(6-ethyl-aminohexylamino) lepidine dihydrochloride, hemihydrate) and WR 254,421 (8-(6'-N,N-diethylaminohexyl)amino-4-hydroxymethyl-6-methoxyquinoline, dihydrochloride) in urine. WR 211,789 has been detected in a rat microsomal preparation.[21] This assay adds the capability of detection of WR 6026, WR 211,789 and WR 254,421 (as free bases) in urine to earlier assays for WR 6026 and WR 211,789 in plasma and blood.

Assay samples were prepared by spiking known volumes of human urine with a known amount (constant over all samples) of the verapamil internal standard (IS). Standard curve samples were generated by spiking interference free human urine samples with known amounts of WR 6026, WR 211,789 and WR 254,421 (as free bases) and IS. These standard curve and assay samples were extracted, then injected onto an HPLC column for separation and subsequent ultraviolet detection. The peak height ratios of WR 6026, WR 211,789 and WR 254,421 (as free bases) to IS were calculated for each sample from the measured peak heights obtained by HPLC. Finally, standard curve concentrations and WR 6026, WR 211,789 and WR 254,421 (as free bases) to IS peak height ratios of the standard curve samples were fit by least squares linear regression to the equation for the best straight line ($y = mx + b$, where y = peak height ratio and x = WR 6026, WR 211,789 or WR 254,421 (as free bases) concentrations), and drug concentrations in assay samples were calculated by this equation from the WR 6026, WR 211,789 and WR 254,421 (as free bases) to IS peak height ratios obtained by HPLC.

Sample volume taken for analysis was 0.5 ml of urine. A constant amount, approximately 5 µg, of the internal standard, verapamil, was added to and mixed with each sample. Next, 100 µl of 1 N NaOH was added to and mixed with each sample. Then, samples were extracted with 5 ml of methyl *t*-butyl ether. The extraction solution was transferred to a second culture tube, evaporated to dryness under nitrogen, and reconstituted in 200 µl of mobile phase. Finally 20-160 µl of the sample was injected onto the HPLC column.

In typical chromatograms for blank urine and urine samples spiked with WR 6026, WR 211,789 or WR 254,421, WR 6026, WR 211,789 or WR 254,421 eluted at 15.3, 14.3, and 18.2 minutes, respectively, and the internal standard eluted at 12.4

minutes. The coefficients of determination for WR 6026, WR 211,789 or WR 254,421 interday and intraday precision standard curves were 0.9825 or higher.

The reversed-phase system (alkyl bonded silica gel with an aqueous mobile phase) is the most widely used HPLC technique in assays for drugs in biological fluids. In this kind of a system, the retention mechanism depends mainly on the lipophilic character of substances to be analyzed. Such a mechanism also retains considerable amounts of other lipophilic substances, thereby interfering with the drug peak. On the other hand, in a system consisting of a bare silica gel and an aqueous mobile phase, the retention mechanism results mainly from ion exchange and only partially from lipophilic interactions. Thus, endogenous non-ionic neutral lipid compounds and anionic compounds will not be retained on the silica gel column; only the cationic (e.g. ammonium) ions will be retained. The interfering substances in biological fluids elute at the solvent front, leaving a very clean baseline around the retention time of the drug.

HPLC assays for basic amine drugs in biological samples that make use of a silica gel column and an aqueous mobile phase have been operated in this laboratory for over 5 years. By use of an organic solvent extraction step for sample clean-up, an unbonded silica gel column combined with an aqueous mobile phase for separation, and the superior capability of ultraviolet detection, the free base concentrations of WR 6026, WR 211,789 and WR 254,421 can be quantitatively and reliably measured in human urine samples. The assay described in the report dated July, 18, 1994, requires 0.5 ml urine samples to determine the free base concentrations of WR 6026, WR 211,789 or WR 254,421. The method involves sample cleanup with a methyl *t*-butyl ether extraction, separation on an unbonded silica gel column (5 μm particle size) run with an aqueous mobile phase, and ultraviolet detection. The minimum quantitation limits of the assay are 5.17, 5.09, and 45.4 ng/ml for WR 6026, WR 211,789 and WR 254,421 free base, respectively, with a signal to noise ratio of 3 to 1. Average mean recoveries over the working range of the standard curve were 97.2, 92.8, and 94.2 percent for WR 6026, WR 211,789 and WR 254,421 free base, respectively. The respective percent coefficients of variation (CVs) of the inter- and intraday assay precision analysis for the free base concentrations of WR 6026 ranged from 3.90% to 7.42% and 3.83% to 28.4%; of WR 211,789 ranged from 4.07% to 10.0% and 5.12% to 23.3%; and of WR 254,421 ranged from 3.09% to 5.86% and 3.55% to 10.0%. No discernible pattern of degradation was observed in long term or autosampler stability tests.

Study Report 26: WR 242511 in Human and Dog Plasma

Study Characteristics: Study Report 26

Test Article:	WR 242511
Test System:	human plasma dog plasma
Internal Standard:	Chlorpheniramine maleate
Sample Assay Volume:	0.5 ml
Sample Cleanup:	methyl *t*-butyl ether extraction

Analytical System

Detector:	UV at 350 nm
Column Type:	silica
Column Size:	4.6x250 mm, 5µ particle size
Mobile Phase:	CH_3CN/H_2O (7:3, v/v) with 0.008% TEA and 0.005% H_3PO_4 (final concentrations)

Validation Results: WR 242511 free base in human plasma

Lower Limit of Quantitation:	4.00 ng/ml
Interday Mean, CV and RE:	4.57 ng/ml, 5.84% and 14.2%
Intraday Mean, CV and RE:	3.69 ng/ml, 7.87% and -15.6%
Standard curve range:	4.00 to 1024 ng/ml
Interday Precision Concentrations:	8.00, 32.0, 128, and 256 ng/ml
CV Range:	8.74 to 11.9%
RE Range:	-3.32 to +5.40%
Intraday Precision Concentrations:	8.00, 32.0, 128, and 256 ng/ml
CV Range:	2.99 to 5.90%
RE Range:	+5.21 to +12.3%
Blind Sample Assay	
Concentration Range:	4.70 to 822 ng/ml
RE Range:	-6.27 to +21.9%
Overall Mean Recovery:	77.1%
Stability	
Plasma Freezer Storage:	-70°C for 6 months -20°C for 6 months
Processed Sample:	Room temp. for 4 days
Plasma Storage:	Room temp. for 6 hours
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	6 months

Short Validation Results: WR 242511 free base in dog plasma

Lower Limit of Quantitation:	4.00 ng/ml
Precision Mean, CV and RE:	4.42 ng/ml, 6.52% and +10.4%
Standard curve range:	4.00 to 1024 ng/ml
Interday Precision Concentrations:	8.00, 32.0, 128, and 256 ng/ml
CV Range:	4.14 to 13.3%
RE Range:	-5.78 to -0.104%
Intraday Precision Concentrations:	8.00, 32.0, 128, and 256 ng/ml
CV Range:	0.764 to 5.12%
RE Range:	-10.8 to -3.35%
Overall Mean Recovery:	79.3%

Study Description: WR 242511 in Human and Dog Plasma (the methodology was presented in DAMD17-92-C-2028 final report)

This report describes a high performance liquid chromatographic (HPLC) assay and provides data validating the assay for a compound of the 8-aminoquinoline class. The compound, 8-[(4-amino-1-methylbutyl)amino]-5-(1-hexyloxy)-6-methoxy-4-methylquinoline (DL) tartrate (WR 242511, Figure 1), holds promise[35] in an effort to replace primaquine, the radical cure and prophylaxis for vivax and ovale malaria and is being developed by WRAIR as an anti-cyanide drug.

Assays for other 8-aminoquinolines include high performance liquid chromatography (HPLC) methods with electrochemical,[36] ultraviolet,[37] and fluorescence[38] detection. An HPLC method with oxidative electrochemical detection has been described[39] for WR 242511 in 0.25 ml plasma samples with a detection limit of 10 ng/ml. This report presents validation data for a superior method that employs an aqueous mobile phase, an unbonded silica gel column[40] and ultraviolet detection for WR 242511 free base concentration determinations in 0.5 ml human and dog plasma samples with a lower limit of quantitation of 4 ng/ml.

Plasma samples were analyzed for WR 242511 free base with an HPLC procedure that uses a silica gel column, an (acetonitrile/water) aqueous mobile phase, UV absorbance detection, and a 0.5 ml method sample size. Sample cleanup consisted of extraction into methyl *t*-butyl ether. The methodology contains detailed procedures, which are summarized below.

Assay samples were prepared by spiking known volumes of human plasma with a known amount (constant over all samples in a run) of CPA internal standard (IS). Standard curve samples were generated by spiking a known amount of WR 242511 tartrate into interference free human plasma which is then brought to a known volume, divided by serial dilution and spiked with a known amount of IS. These standard curve and assay samples were prepared for analysis, then 40 µl aliquots were injected onto the HPLC column for

chromatographic separation and subsequent UV absorbance detection of drug and IS peaks. The peak height ratios of WR 242511 to IS were calculated for each sample from the measured peak heights obtained by HPLC. Next, standard curve concentrations and WR 242511 to IS peak height ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the equation for the best straight line, $y = mx + b$, where y = peak height ratio and x = WR 242511 free base concentrations. Finally, drug concentrations in assay samples were calculated for each run by this equation from the WR 242511 to IS peak height ratios obtained by HPLC.

Stock solutions of WR 242511 tartrate and chlorpheniramine maleate internal standard (IS) were stored in a 4°C refrigerator and protected against exposure to light as necessary, and checked for deterioration by following the ratio of drug to internal standard peak heights in a diluted solution (solutions are discarded when a more than 10% change in the ratio is observed or by 2 months after the preparation date).

Plasma samples for analysis were thawed and mixed by vortexing (if appropriate), then pipetted (0.5 ml) into glass culture tubes. A constant amount (1.0 µg chlorpheniramine maleate) of IS, 100 µl of 0.1N NaOH, and 3 ml of methyl *t*-butyl ether are added. Upon centrifugation and freezing of the aqueous layer, the resulting supernatant was transferred to a clean tube, evaporated to dryness, reconstituted in 70% acetonitrile, transferred to WISP vials, and injected onto the column.

The assay described in the report dated December, 12, 1996, requires 0.5 ml plasma samples to determine the concentrations of WR 242511 free base. The method involves extraction from plasma with methyl *t*-butyl ether, separation on a silica gel column with an aqueous mobile phase in an isocratic elution, and ultraviolet absorbance detection. The advantages of this method include a clean baseline and a short run time.

The reversed-phase system (alkyl bonded silica gel with an aqueous mobile phase) is the most widely used HPLC technique in assays for drugs in biological fluids. In this kind of a system, the retention mechanism depends mainly on the lipophilic character of substances to be analyzed. Such a mechanism also retains considerable amounts of other lipophilic substances, thereby interfering with the drug peak. On the other hand, in a system consisting of a bare silica gel and an aqueous mobile phase, the retention mechanism results mainly from ion exchange[6] and only partially from lipophilic interactions. Thus, endogenous non-ionic neutral lipid compounds and anionic compounds will not be retained on the silica gel column; only the cationic (e.g. ammonium) ions will be retained. The interfering substances in biological fluids elute at the solvent front, leaving a very clean baseline around the retention time of the drug. In this method, the mobile phase is recycled through a non analytical silica gel column overnight to saturate it with silica. Overnight saturation of mobile phase prior to use is beneficial to the whole system, since silica gel slowly dissolves in neutral aqueous solution and the water flowing through the silica gel approaches the equilibrium concentration of silica. The saturated mobile phase does not

dissolve silica from the analytical columns and degradation of the analytical column is decreased relative to the single pass system.

By use of a solvent extraction step for sample clean-up, an unbonded silica gel column combined with an aqueous mobile phase for separation, and the superior capability of ultraviolet detection, the concentration of WR 242511 free base can be quantitatively and reliably measured in human and dog plasma samples. The drug and IS are baseline separated, and no interfering peaks were observed. The assay was demonstrated to be linear within the range of the standard curve, 4.00 to 1024 ng/ml WR 242511 free base. The CVs of results for human plasma precision validation ranged from 8.74 to 11.9% interday and 2.99 to 5.90% intraday, while percent RE of measured results compared to serially diluted concentrations ranged -3.32 to +5.40% interday and +5.21 to +12.3% intraday for WR 242511 free base. The mean concentrations (n = 6) obtained for human plasma samples serially diluted to the LLOQ (4.00 ng/ml) were 4.57 ng/ml interday (5.84% CV and +14.2% RE) and 3.38 ng/ml intraday (7.87% CV and -15.6% RE) where the signal to noise ratio was better than 3 to 1. WR 242511 average recovery from human plasma extraction for the four concentrations within the standard curve quantitation limits was 77.1%. Stability test results indicate WR 242511 is sufficiently stable in 1) human plasma samples prepared for assay (includes extraction, evaporation, and reconstitution in 70% acetonitrile) to withstand room temperature (RT) storage for at least 4 days, 2) human plasma at -70°C and at -20°C to permit storage without significant degradation for up to 6 months, 3) human plasma to withstand RT storage for at least 6 hours without significant degradation, and 4) human plasma to withstand 5 cycles of repeated freezing in a -70°C freezer and thawing at room temperature without significant degradation. The CVs of the results for the analyses of blind WR 242511 human plasma samples (n = 5) at five concentrations within the standard curve limits ranged 0.958-9.61% while R.E.s ranged -8.39 to +21.9%.

The CV (and corresponding RE, for n = 6) results of dog plasma precision validation for WR 242511 ranged from 4.14 to 13.3% (-5.78 to -0.104%) interday and 0.764 to 5.12% (-10.8 to -3.35%) intraday. Mean back calculated dog plasma concentration results of replicate analyses of precision standard curve samples serially diluted to the LLOQ (4.00 ng/ml for WR 242511 free base concentration) was 4.42 ng/ml (6.52% CV and +10.4% RE, n = 4). The signal to noise ratio was better than 3 to 1 for these LLOQ samples. WR 242511 average recovery from dog plasma extraction for the four concentrations within the standard curve quantitation limits was 79.3%.

Study Report 28: Halofantrine and WR 178460 R&S Isomers in Human Plasma

Study Characteristics: Study Report 28

Test Article:	Halofantrine (R isomer) Halofantrine (S isomer) WR 178460 (R isomer) WR 178460(S isomer)
Test System:	human plasma

This project was requested as described in a COR letter dated Oct. 20, 1995. WR 216062 and WR 216063 standard samples were received October 20, 1995 for use in development and validation of an assay for halofantrine and desbutylhalofantrine enantiomers in human plasma. A draft report is in preparation.

Study Report 29A: Chloroquine and Monodesethylchloroquine in Human Blood

Study Characteristics: Study Report 29A

Test Article:	Chloroquine Monodesethylchloroquine
Test System:	human blood
Internal Standard:	Neostigmine bromide
Sample Assay Volume:	100 µl
Sample Cleanup:	Lyse cells with water, precipitate proteins with acetonitrile

Analytical System

Detector:	MS/MS
Column Type:	Hypersil silica
Column Size:	4.6x50 mm, 3 µ particle size
Mobile Phase:	CH_3CN/H_2O (9:1, v/v) with 0.1% TFA and 5mM ammonium acetate

Validation Results: Chloroquine free base in human blood

Lower Limit of Quantitation:	20 ng/ml
Interday Mean, CV and RE:	16.9 ng/ml, 5.93% and –15.4%
Intraday Mean, CV and RE:	22.6 ng/ml, 9.81% and +12.9%
Standard curve range:	20 to 2000 ng/ml
Interday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	7.57% to 11.4%
RE Range:	-7.49 to –1.99 %
Intraday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	2.04 to 8.22 %
RE Range:	-8.08 to +0.210%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	70.4%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	4°C for 27 days
Plasma Storage:	Room temp. for 6 hours
5 Cycle Freeze/Thaw:	5 cycles to -20°C
Standard Solution:	Not determined

Validation Results: Monodesethylchloroquine free base in human blood

Lower Limit of Quantitation:	20 ng/ml
Interday Mean, CV and RE:	17.8 ng/ml, 11.0%, and -10.8
Intraday Mean, CV and RE:	22.2 ng/ml, 8.34%, and +11.2%
Standard curve range:	20 to 2000 ng/ml
Interday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	8.51% to 13.0%
RE Range:	-8.28% to -4.87%
Intraday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	2.62% to 9.84%
RE Range:	-11.4% to +0.103%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	61.3%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	4°C for 27 days
Plasma Storage:	Room temp. for 6 hours
5 Cycle Freeze/Thaw:	5 cycles to -20°C
Standard Solution:	Not determined

This project was requested in a COR letter dated December 6, 1995. An interim report was submitted for review August 22, 1997 for a chloroquine/monodesethylchloroquine method for human blood. A December 8, 1997, fax from the COR requested changes in the interim report which will be incorporated into final Study Report 29A.

This report describes the analytical method and validation of the analytical method used to measure concentrations of chloroquine and monodesethyl-chloroquine in human blood samples. The method was developed and validated at the Analytical Division, Drug Studies Unit (DSU), UCSF, San Francisco, CA. The analysis of human blood samples was accomplished by use of the liquid chromatographic/mass spectrometric/mass spectrometric (LC/MS/MS) method.

Method Summary: Human blood samples (100 µl) were analyzed for chloroquine free base (CHL), and monodesethyl-chloroquine (MDC) with an LC/MS/MS procedure in a PE Sciex-API III® system that uses a silica gel column, an acetonitrile/water/TFA (90:10:0.1, v/v) with 5 mM ammonium acetate mobile phase, and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (Atmospheric Pressure Chemical Ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample cleanup consisted of addition of water and sonication to lyse cells, precipitation with a neostigmine internal standard solution in acetonitrile, centrifugation and transfer of the supernatant prior to separation by LC/MS/MS.

Validation Results Summary: The following validation parameters were evaluated for CHL and MDC.

1. Specificity: No significant endogenous interfering peaks for CHL, MDC, or for the internal standard were observed in blank human blood.

2. Inter-Day Precision and Accuracy: Interday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of human blood spiked with known amounts of drug and metabolite. Each of 6 sets (n=2) of control samples at 4 different drug and metabolite concentrations was evaluated (6 standard curves for the drug and metabolite were run). Precision coefficients of variation (CV), ranged from 7.57% to 11.4% for CHL and from 8.51% to 13.0% for MDC. The accuracy, defined by the relative error (RE) ranged from -7.49% to -1.99% for CHL and from -8.28% to -4.87% for MDC.

3. Intra-Day Precision and Accuracy: Intraday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of human blood spiked with known amounts of drug and metabolite. For intraday precision, 6 sets (n=1) of control samples for each of 4 different drug and metabolite concentrations were evaluated with 1 standard curve on the same run. CVs ranged from 2.04% to 8.22% for CHL and from 2.62% to 9.84% for MDC. R.E.s ranged from -8.08% to +0.210% for CHL and from -11.4% to +0.103% for MDC.

4. Lower Limit of Quantitation (LLOQ): The LLOQs for this assay are equivalent to the low points of the standard curves, or 20.0 ng/ml each for CHL and MDC. Interday mean, CV, and RE results are 16.9 ng/ml, 5.93%, and -15.4% for CHL and 17.8 ng/ml, 11.0%, and -10.8 for MDC. Intraday mean, CV, and RE results are 22.6 ng/ml, 9.81%, and +12.9% for CHL and 22.2 ng/ml, 8.34%, and +11.2% for MDC.

5. Linear Range: The validated linear concentration ranges for this assay were 20.0 to 2000 ng/ml for CHL and MDC.

6. Recovery: Peak area ratios of processed samples to unprocessed samples provided overall recoveries of 70.4% for CHL and 61.3% for MDC.

7. Stability:

    a. Freeze/Thaw: CHL and MDC were shown to be stable in human blood for up to 5 freeze/thaw cycles when samples are frozen to -20°C and thawed to room temperature.

    b. Bench Top: CHL and MDC were shown to be stable for at least 6 hours in human blood at ambient temperature.

c. Processed Samples: CHL, MDC, and internal standard were shown to be stable up to 27 days at 4°C. A test at the ambient temperature is yet to be performed.

d. Long Term: A test of stability of -20°C freezer storage is yet to be performed.

Human blood samples (100 µl) were analyzed for chloroquine and monodesethyl-chloroquine with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a silica column (4.6 x 50 mm, 5 µm particle size), 90% CH_3CN, 0.1% trifluoroacetic acid (TFA) and (final concentration) 5 mM ammonium acetate mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (atmospheric pressure chemical ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of 50 µl of water, sonication of the mixture, addition of neostigmine bromide internal standard (IS), and transfer of the supernatant prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free human blood samples with known amounts of chloroquine, monodesethyl-chloroquine, and IS. Standard curve, QC and assay samples were prepared as described, then 1-2 µl aliquots were injected into the LC/MS/MS system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios of chloroquine and monodesethyl-chloroquine to IS were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations and chloroquine and monodesethyl-chloroquine to IS peak area ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the two equations for the best straight lines ($y = mx + b$, where y = peak area ratio and x = chloroquine or monodesethyl-chloroquine concentrations), and drug and metabolite concentrations in assay samples were calculated by these equations from the chloroquine and monodesethyl-chloroquine to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank human blood with chloroquine and monodesethyl-chloroquine. A didesethyl-chloroquine stock solution was also generated, diluted into working solutions and spiked into validation samples but data is not presented in this report, since validation acceptance criteria were not met.

Conclusion: The LC/MS/MS method for analysis of human blood to determine concentrations of CHL and MDC was validated for the concentration ranges of 20.0 ng/ml to 2000 ng/ml for CHL and MDC. The method was demonstrated to be precise, accurate, and sufficiently reproducible for analysis of study samples.

Study Report 29B: Chloroquine, Monodesethylchloroquine and Didesethylchloroquine in Human Blood and Plasma

Study Characteristics: Study Report 29B

Test Article:	Chloroquine Monodesethylchloroquine Didesethylchloroquine
Test System:	human blood human plasma
Internal Standard:	Neostigmine bromide
Sample Assay Volume:	100 µl
Sample Cleanup:	Blood: Lyse cells with water Blood and plasma: precipitate proteins with acetonitrile

Analytical System

Detector:	MS/MS
Column Type:	Hypersil silica
Column Size:	4.6x50 mm, 3 µ particle size
Mobile Phase:	CH_3CN/H_2O (9:1, v/v) with 0.1% TFA and 5mM ammonium acetate

Validation Results: Chloroquine free base in human blood

Lower Limit of Quantitation:	20 ng/ml
Interday Mean, CV and RE:	20.0 ng/ml, 8.26%, and –0.250%
Intraday Mean, CV and RE:	16.7 ng/ml, 7.06%, and –16.7%
Standard curve range:	20 to 2000 ng/ml
Interday Precision Concentrations:	40, 500, and 1500 ng/ml
CV Range:	2.57% to 5.67%
RE Range:	–2.56% to +4.39%
Intraday Precision Concentrations:	40, 500, and 1500 ng/ml
CV Range:	1.50% to 5.53%
RE Range:	–4.29% to +7.78%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	63.1%
Stability	
Plasma Freezer Storage:	10 months at -70°C
Processed Sample:	8.5 hours at ambient temp. 4°C for 31 days
Plasma Storage:	6 hours at ambient temp.
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	Not determined

Validation Results: Monodesethylchloroquine free base in human blood

Lower Limit of Quantitation:	20 ng/ml
Interday Mean, CV and RE:	19.8 ng/ml, 9.88%, and –1.17%
Intraday Mean, CV and RE:	16.1 ng/ml, 8.09%, and –19.4%
Standard curve range:	20 to 2000 ng/ml
Interday Precision Concentrations:	40, 500, and 1500 ng/ml
CV Range:	2.97% to 4.92%
RE Range:	–6.22% to +2.22%
Intraday Precision Concentrations:	40, 500, and 1500 ng/ml
CV Range:	2.31% to 10.8%
RE Range:	-6.00% to +12.3%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	41.5%
Stability	
Plasma Freezer Storage:	10 months at -70°C
Processed Sample:	8.5 hours at ambient temp. 4°C for 31 days
Plasma Storage:	6 hours at ambient temp.
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	Not determined

Validation Results: Didesethylchloroquine free base in human blood

Lower Limit of Quantitation:	20 ng/ml
Interday Mean, CV and RE:	21.6 ng/ml, 8.39%, and +7.75%
Intraday Mean, CV and RE:	17.9 ng/ml, 15.0%, and –10.67%
Standard curve range:	20 to 2000 ng/ml
Interday Precision Concentrations:	40, 500, and 1500 ng/ml
CV Range:	1.94% to 6.29%
RE Range:	-10.5% to +4.56%
Intraday Precision Concentrations:	40, 500, and 1500 ng/ml
CV Range:	2.26% to 7.43%
RE Range:	-11.0% to +13.0%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	54.6%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	8.5 hours at ambient temp. 4°C for 31 days
Plasma Storage:	6 hours at ambient temp.
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	Not determined

Validation Results: Chloroquine free base in human plasma

Lower Limit of Quantitation:	20 ng/ml
Interday Mean, CV and RE:	17.6 ng/ml, 2.80%, and –11.8%
Intraday Mean, CV and RE:	Not determined
Standard curve range:	20 to 2000 ng/ml
Interday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	3.60% to 8.12%
RE Range:	–2.33% to +10.1%
Intraday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	1.28% to 6.68%
RE Range:	–11.3% to +4.00%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	88.1%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	Not determined
Plasma Storage:	Not determined
5 Cycle Freeze/Thaw:	Not determined
Standard Solution:	Not determined

Validation Results: Monodesethylchloroquine free base in human plasma

Lower Limit of Quantitation:	20 ng/ml
Interday Mean, CV and RE:	19.0 ng/ml, 3.80%, and –5.00%
Intraday Mean, CV and RE:	Not determined
Standard curve range:	20 to 2000 ng/ml
Interday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	2.02% to 7.67%
RE Range:	–4.21% to +9.93%
Intraday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	2.22% to 3.66%
RE Range:	-10.0% to +7.07%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	88.1%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	Not determined
Plasma Storage:	Not determined
5 Cycle Freeze/Thaw:	Not determined
Standard Solution:	Not determined

Validation Results: Didesethylchloroquine free base in human plasma

Lower Limit of Quantitation:	20 ng/ml
Interday Mean, CV and RE:	20.2 ng/ml, 10.8%, and +1.17%
Intraday Mean, CV and RE:	Not determined
Standard curve range:	20 to 2000 ng/ml
Interday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	2.31% to 11.3%
RE Range:	-1.57% to +6.40%
Intraday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	1.12% to 13.9%
RE Range:	-5.17% to +5.07%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	83.5%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	Not determined
Plasma Storage:	Not determined
5 Cycle Freeze/Thaw:	Not determined
Standard Solution:	Not determined

Study Description: Study Report 29B on validation of a method for chloroquine, monodesethylchloroquine and didesethylchloroquine in human blood and including a short validation in human plasma is in preparation.

Human Blood

This report describes the analytical method and validation of the analytical method used to measure concentrations of chloroquine (and its metabolites) in human blood samples. The method was developed and validated at the Analytical Division, Drug Studies Unit (DSU), UCSF, San Francisco, CA. The method was requested by the Contracting Officer's Representative at the Walter Reed Army Institute of Research (WRAIR) for contract DAMD17-97-C-7058.

Method Summary: Human blood samples (100 µl) were analyzed for chloroquine (and its metabolites) with an LC/MS/MS procedure in a PE Sciex-API III® triple quadrupole system that uses a silica gel column, an acetonitrile/water/TFA (90:10:0.1, v/v) with 5 mM ammonium acetate mobile phase, and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (Atmospheric Pressure Chemical Ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample cleanup consisted of addition of water, sonication, precipitation with a neostigmine internal standard solution in acetonitrile, centrifugation and transfer of the supernatant prior to separation by LC/MS/MS.

Validation Results Summary: The following validation parameters were evaluated for chloroquine (CHL) and its metabolites monodesethyl-chloroquine (MCL) and didesethylchloroquine (DCL).

1. Specificity: No significant endogenous interfering peaks for chloroquine (or its metabolites), quinine or for the internal standard were observed in blank human blood.

2. Inter-Day Precision and Accuracy: Interday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of human blood spiked with known amounts of drug. Each of 6 sets (n=2) of control samples at 3 different drug concentrations was evaluated (6 standard curves for the drug were run). Precision coefficients of variation (CV), ranged from 2.57% to 5.67% for CHL, 1.94% to 6.29% for DCL, and 2.97% to 4.92% for MCL. The accuracy, defined by the relative error (RE) ranged from –2.56% to +4.39% for CHL, -10.5% to +4.56% for DCL, and –6.22% to +2.22% for MCL.

3. Intra-Day Precision and Accuracy: Intraday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of human blood spiked with known amounts of drug. For intraday precision, 6 sets (n=1) of control samples for each of three different drug concentrations were evaluated with one standard curve on the same run. CVs ranged from 1.50% to 5.53% for CHL, 2.26% to 7.43% for DCL, and 2.31% to 10.8% for MCL. The REs ranged from –4.29% to +7.78% for CHL, -11.0% to +13.0% for DCL, -6.00% to +12.3% for MCL.

4. Lower Limit of Quantitation (LLOQ): The LLOQs for this assay are equivalent to the low points of the standard curves, or 20.0 ng/ml for CHL, and DCL. Interday mean, CV, and RE results are 20.0 ng/ml, 8.26%, and –0.250% for CHL, 21.6 ng/ml, 8.39%, and +7.75% for DCL, and 19.8 ng/ml, 9.88%, and –1.17% for MCL. Intraday mean, CV, and RE results are 16.7 ng/ml, 7.06%, and –16.7% for CHL, 17.9 ng/ml, 15.0%, and –10.67% for DCL, and 16.1 ng/ml, 8.09%, and –19.4% for MCL.

5. Linear Range: The validated linear concentration ranges for this assay were 20.0 to 2000 ng/ml for CHL, DCL, and MCL.

6. Recovery: Peak area ratios of processed samples to unprocessed samples provided overall recoveries of 63.1% for CHL, 41.5% for DCL, and 54.6% for MCL.

7. Stability:

   a. Freeze/Thaw: CHL, DCL, and MCL were shown to be stable in human blood for up to 5 freeze/thaw cycles when samples are frozen to -70°C and thawed to room temperature.

   b. Bench Top: CHL, DCL, and MCL were shown to be stable for at least 6 hours in human blood at ambient temperature.

c. Processed Sample – Ambient temperature: CHL, DCL, MCL, and internal standard were shown to be stable up to 8.5 hours at ambient temperature.

Processed Sample - Refrigerated: CHL, DCL, MCL, and internal standard were shown to be stable up to 31 days at 4°C.

d. Long Term: CHL, DCL, and MCL were shown to be stable for up to 10 months in human blood at -70°C.

Method: Human blood samples (100 µl) were analyzed for chloroquine, monodesethylchloroquine, and didesethylchloroquine with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a silica column (4.6 x 50 mm, 3 µm particle size), 90% CH_3CN, 0.1% trifluoroacetic acid (TFA) and 5 mM ammonium acetate mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (atmospheric pressure chemical ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of 50 µl of water, sonication of the mixture for 5 minutes, addition of 400 µl of acetonitrile containing neostigmine internal standard (IS), mixing of the mixture for 1 minute, centrifugation for 10 minutes and transfer of the supernatant prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free human blood samples with known amounts of chloroquine monodesethylchloroquine, didesethylchloroquine, and IS. Standard curve, QC and assay samples were prepared as described, then ~2 µl aliquots were injected into the LC/MS/MS system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios of chloroquine (daughter ion at 247 *m/z* from parent ion at 321 *m/z*) and monodesethylchloroquine (daughter ion at 114 *m/z* from parent ion at 292 *m/z*), didesethylchloroquine (daughter ion at 179 *m/z* from parent ion at 265 *m/z*), (daughter ion at 251 *m/z* from parent ion at 325 *m/z*) to IS (daughter ion at 72 *m/z* from parent ion at 209 *m/z*) were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations of chloroquine, monodesethyl-chloroquine, and didesethylchloroquine to IS peak area ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the three equations for the best straight lines ($y = mx + b$, where y = peak area ratio and x = chloroquine, monodesethyl-chloroquine, or didesethylchloroquine concentrations), and drug and metabolite concentrations in assay samples were calculated by these equations from the chloroquine, monodesethylchloroquine, and didesethylchloroquine to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank human blood with chloroquine, monodesethyl-chloroquine, and didesethylchloroquine.

Retention times (approximate, in minutes) were internal standard 1:22, chloroquine 1:54, monodesethylchloroquine 1:40, didesethylchloroquine 1:40. The time between injections was 2-3 minutes.

Conclusion: The LC/MS/MS method for analysis of human blood to determine concentrations of chloroquine (and its metabolites) was validated for the concentration ranges of 20.0 ng/ml to 2000 ng/ml for chloroquine monodesethylchloroquine, and didesethylchloroquine. "Preparation time for a run of 40 sponsor samples plus standard curve and control samples is about 1&1/2 hours. Run time is about 3&1/4 hours." The method was demonstrated to be precise, accurate, and sufficiently reproducible for analysis of study samples.

Human Plasma

This report describes the analytical method and validation of the analytical method used to measure concentrations of chloroquine (and its metabolites) in human plasma samples. The method was developed and validated at the Analytical Division, Drug Studies Unit (DSU), UCSF, San Francisco, CA. The method was requested by the Contracting Officer's Representative at the Walter Reed Army Institute of Research (WRAIR) for contract DAMD17-97-C-7058.

Method Summary: Human plasma samples (100 µl) were analyzed for chloroquine (and its metabolites) with an LC/MS/MS procedure in a PE Sciex-API III® triple quadrupole system that uses a silica gel column, an acetonitrile/water/TFA (90:10:0.1, v/v) with 5 mM ammonium acetate mobile phase, and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (Atmospheric Pressure Chemical Ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample cleanup consisted of precipitation with a neostigmine internal standard solution in acetonitrile, centrifugation and transfer of the supernatant prior to separation by LC/MS/MS.

Validation Results Summary: The following validation parameters were evaluated for chloroquine (CHL) and its metabolites monodesethyl-chloroquine (MCL) and didesethylchloroquine (DCL).

1. Specificity: No significant endogenous interfering peaks for chloroquine (or its metabolites), quinine or for the internal standard were observed in blank human plasma.

2. Inter-Day Precision and Accuracy: Interday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of human plasma spiked with known amounts of drug. Each of 3 sets (n=2) of control samples at 4 different drug concentrations was evaluated (3 standard curves for the drug were run). Precision coefficients of variation (CV), ranged from 3.60% to 8.12% for CHL, 2.31% to 11.3% for DCL, and 2.02% to 7.67% for MCL. The accuracy, defined by the relative

error (RE) ranged from –2.33% to +10.1% for CHL, -1.57% to +6.40% for DCL, and –4.21% to +9.93% for MCL.

3. Intra-Day Precision and Accuracy: Intraday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of human plasma spiked with known amounts of drug. For intraday precision, 6 sets (n=1) of control samples for each of four different drug concentrations were evaluated with one standard curve on the same run. CVs ranged from 1.28% to 6.68% for CHL, 1.12% to 13.9% for DCL, and 2.22% to 3.66% for MCL. The REs ranged from –11.3% to +4.00% for CHL, -5.17% to +5.07% for DCL, -10.0% to +7.07% for MCL.

4. Lower Limit of Quantitation (LLOQ): The LLOQs for this assay are equivalent to the low points of the standard curves, or 20.0 ng/ml for CHL, and DCL. Interday mean, CV, and RE results are 17.6 ng/ml, 2.80%, and –11.8% for CHL, 20.2 ng/ml, 10.8%, and +1.17% for DCL, and 19.0 ng/ml, 3.80%, and –5.00% for MCL.

5. Linear Range: The validated linear concentration ranges for this assay were 20.0 to 2000 ng/ml for CHL, DCL, and MCL.

6. Recovery: Peak area ratios of processed samples to unprocessed samples provided overall recoveries of 88.1% for CHL, 83.5% for DCL, and 88.1% for MCL.

Method: Human plasma samples (100 µl) were analyzed for chloroquine, monodesethylchloroquine, and didesethylchloroquine with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a silica column (4.6 x 50 mm, 3 µm particle size), 90% CH_3CN, 0.1% trifluoroacetic acid (TFA) and 5 mM ammonium acetate mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (atmospheric pressure chemical ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of 400 µl of acetonitrile containing neostigmine internal standard (IS), mixing of the mixture for 1 minute, centrifugation for 5 minutes and transfer of the supernatant prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free human plasma samples with known amounts of chloroquine monodesethylchloroquine, didesethylchloroquine, and IS. Standard curve, QC and assay samples were prepared as described, then ~2 µl aliquots were injected into the LC/MS/MS system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios of chloroquine (daughter ion at 247 *m/z* from parent ion at 321 *m/z*) and monodesethylchloroquine (daughter ion at 114 *m/z* from parent ion at 292 *m/z*), didesethylchloroquine (daughter ion at 179 *m/z* from parent ion at 265 *m/z*), (daughter ion at 251 *m/z* from parent ion at 325 *m/z*) to IS (daughter ion at 72 *m/z* from parent ion at 209 *m/z*) were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations of chloroquine, monodesethyl-chloroquine, and didesethylchloroquine to IS peak area ratios of the standard curve samples were fit by 1/y weighted least squares

linear regression to the three equations for the best straight lines (y = mx + b, where y = peak area ratio and x = chloroquine, monodesethyl-chloroquine, or didesethylchloroquine concentrations), and drug and metabolite concentrations in assay samples were calculated by these equations from the chloroquine, monodesethylchloroquine, and didesethylchloroquine to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration ranges of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank human plasma with chloroquine, monodesethyl-chloroquine, and didesethylchloroquine.

Retention times (approximate, in minutes) were internal standard 1:23, chloroquine 1:57, monodesethylchloroquine 1:44, didesethylchloroquine 1:45. The time between injections was 2-3 minutes.

Conclusion: The LC/MS/MS method for analysis of human plasma to determine concentrations of chloroquine (and its metabolites) was validated for the concentration ranges of 20.0 ng/ml to 2000 ng/ml for chloroquine monodesethylchloroquine, and didesethylchloroquine. "Preparation time for a run of 40 sponsor samples plus standard curve and control samples is about 1&1/2 hours. Run time is about 3&1/4 hours." The method was demonstrated to be precise, accurate, and sufficiently reproducible for analysis of study samples.

Study Report 30: WR 243251 in Human Plasma

Study Characteristics: Study Report 30

Test Article:	WR 243251
Test System:	human plasma
Analytical System	
Detector:	MS/MS

This project was requested in a COR letter dated May 6, 1996. WR 243,251 standard compound was received May 10, 1996. Method development is in progress.

Study Report 31: WR 238,608, Mefloquine, Chloroquine, Quinine, Doxycycline and Halofantrine in Dog Plasma

Part I: WR 238,608 and Mefloquine

Study Characteristics: Study Report 31, Part 1

Test Article:	WR 238,608 Mefloquine
Test System:	dog plasma
Internal Standard:	Verapamil
Sample Assay Volume:	200 µl
Sample Cleanup:	Precipitate proteins with methanol and acetonitrile

Analytical System

Detector:	MS/MS
Column Type:	Hypersil silica
Column Size:	4.6x50 mm, 3 µ particle size
Mobile Phase:	CH_3CN/H_2O (9:1, v/v) with 0.06% TFA

Validation Results: WR 238,608 free base in dog plasma

Lower Limit of Quantitation:	4.00 ng/ml
Interday Mean, CV and RE:	3.93 ng/ml, 11.5% and –1.75%
Intraday Mean, CV and RE:	3.74 ng/ml, 11.2% and -6.46%
Standard curve range:	4.00 to 800 ng/ml
Interday Precision Concentrations:	8, 40, 160, and 600 ng/ml
CV Range:	4.20% to 9.64%
RE Range:	-1.99% to +6.35%
Intraday Precision Concentrations:	8, 40, 160, and 600 ng/ml
CV Range:	4.64% to 13.2%
RE Range:	-4.75% to +6.46%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	82.4%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	Room Temp. for 3 hours 4°C for 4 days
Plasma Storage:	Room temp. for 6 hours
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	Not determined

Validation Results: Mefloquine free base in dog plasma

Lower Limit of Quantitation:	5.00 ng/ml
Interday Mean, CV and RE:	4.90 ng/ml, 9.66%, and -2.10%
Intraday Mean, CV and RE:	4.47 ng/ml, 14.1%, and -4.60%
Standard curve range:	5.00 to 1000 ng/ml
Interday Precision Concentrations:	10, 50, 200, and 750 ng/ml
CV Range:	3.56% to 8.90%
RE Range:	-3.31% to +3.00%
Intraday Precision Concentrations:	10, 50, 200, and 750 ng/ml
CV Range:	2.78% to 3.56%
RE Range:	-5.28% to +3.17%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	90.2%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	Room Temp. for 3 hours 4°C for 4 days
Plasma Storage:	Room temp. for 6 hours
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	Not determined

Study Description: This validation of methods on WR 238,605 used in combination with mefloquine, chloroquine, halofantrine, quinine and doxycycline in dog plasma was requested as described in a COR letter dated Oct. 20, 1995.

Draft Study Report 31, Part I, on validation of WR 238,605 and mefloquine was submitted for review on December 4, 1998. In response to the review received January 8, 1999, the final Study Report 31, Part I, was submitted May 10, 1999.

This report describes the analytical method and validation of the analytical method used to measure concentrations of WR 238,605 and mefloquine in dog plasma samples. The method was developed and validated at the Analytical Division, Drug Studies Unit (DSU), UCSF, San Francisco, CA. The method was requested by the Contracting Officer's Representative at the Walter Reed Army Institute of Research (WRAIR) for contract DAMD17-97-C-7058 for validation of a method for a study of WR 238,605 used in combination with mefloquine, chloroquine, quinine, halofantrine and doxycycline in dog plasma and subsequent studies.

Method Summary: Dog plasma samples (200 µl) were analyzed for WR 238,605 (free base) and mefloquine (free base) with an LC/MS/MS procedure in a PE Sciex-API III® system that uses a silica gel column, an acetonitrile/water/TFA (90:10:0.06, v/v) mobile phase, and mass spectrometric detection with sample

inlet by heated nebulizer, positive ionization by APCI (Atmospheric Pressure Chemical Ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample cleanup consisted of addition of methanol, precipitation with a verapamil internal standard solution in acetonitrile, centrifugation and transfer of the supernatant prior to separation by LC/MS/MS.

Validation Results Summary: The following validation parameters were evaluated for WR 238,605 and Mefloquine.

1. Specificity: No significant endogenous interfering peaks for WR 238,605, Mefloquine or for the internal standard were observed in blank dog plasma.

2. Inter-Day Precision and Accuracy: Interday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of dog plasma spiked with known amounts of drug. Each of 6 sets (n=2) of control samples at 4 different drug concentrations was evaluated (6 standard curves for the drug were run). Precision coefficients of variation (CV), ranged from 4.20% to 9.64% for WR 238,605 and from 3.56% to 8.90% for Mefloquine. The accuracy, defined by the relative error (RE) ranged from -1.99% to +6.35% for WR 238,605 and from -3.31% to +3.00% for Mefloquine.

3. Intra-Day Precision and Accuracy: Intraday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of dog plasma spiked with known amounts of drug. For intraday precision, 6 sets (n=1) of control samples for each of 4 different drug concentrations were evaluated with 1 standard curve on the same run. CVs ranged from 4.64% to 13.2% for WR 238,605 and from 2.78% to 3.56% for Mefloquine. REs ranged from -4.75% to +6.46% for WR 238,605 and from -5.28% to +3.17% for Mefloquine.

4. Lower Limit of Quantitation (LLOQ): The LLOQs for this assay are equivalent to the low points of the standard curves, or 4.00 ng/ml for WR 238,605 and 5.00 ng/ml Mefloquine. Interday mean, CV, and RE results are 3.93 ng/ml, 11.5%, and -1.75% for WR 238,605 and 4.90 ng/ml, 9.66%, and -2.10 for Mefloquine. Intraday mean, CV, and RE results are 3.74 ng/ml, 11.2%, and -6.46% for WR 238,605 and 4.47 ng/ml, 14.1%, and -4.60% for Mefloquine.

5. Linear Range: The validated linear concentration ranges for this assay were 4.00 to 800 ng/ml for WR 238,605 and 5.00 to 1000 ng/ml for Mefloquine.

6. Recovery: Peak area ratios of processed samples to unprocessed samples provided overall recoveries of 82.4% for WR 238,605 and 90.2% for Mefloquine.

7. Stability:

a. Freeze/Thaw: WR 238,605 and Mefloquine were shown to be stable in dog plasma for up to 5 freeze/thaw cycles when samples are frozen to -70°C and thawed to room temperature.

b. Bench Top: WR 238,605 and Mefloquine were shown to be stable for at least 6 hours in dog plasma at ambient temperature.

c. Processed Sample – Ambient temperature: Mefloquine, WR 238,605 and internal standard were shown to be stable up to 3 hours at ambient temperatures.

Processed Sample - Refrigerated: Mefloquine and internal standard were shown to be stable up to 16 days at 4°C. WR 238,605 was stable up to 4 days at 4°C.

d. Long Term: A freezer (-70°C) 24 month storage stability test is in progress.

Method: Dog plasma samples (200 µl) were analyzed for WR 238,605 and mefloquine with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a silica column (4.6 x 50 mm, 3 µm particle size) and 90% CH_3CN, 0.06% trifluoroacetic acid (TFA) mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (atmospheric pressure chemical ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of 100 µl of methanol, addition of verapamil internal standard (IS), and transfer of the supernatant prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free dog plasma samples with known amounts of WR 238,605, mefloquine, and IS. Standard curve, QC and assay samples were prepared as described, then ~5 µl aliquots were injected into the LC/MS/MS system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios for WR 238,605 (daughter ion at 86 *m/z* from parent ion at 464 *m/z*) and mefloquine (daughter ion at 359 *m/z* from parent ion at 379 *m/z*) to IS (daughter ion at 165 *m/z* from parent ion at 455 *m/z*) were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations and WR 238,605 and mefloquine to IS peak area ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the two equations for the best straight lines ($y = mx + b$, where y = peak area ratio and x = WR 238,605 or mefloquine concentrations), and drug concentrations in assay samples were calculated by these equations from the WR 238,605 and mefloquine to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the

assay. Calibration standards and validation samples were generated by spiking blank dog plasma with WR 238,605 and mefloquine.

Conclusion: The LC/MS/MS method for analysis of dog plasma to determine concentrations of WR 238,605 and Mefloquine was validated for the concentration ranges of 4.00 ng/ml to 800 ng/ml for WR 238,605 and 5.00 ng/ml to 1000 ng/ml for Mefloquine. The method was demonstrated to be precise, accurate, and sufficiently reproducible for analysis of study samples.

Study Report 31, Part II: Chloroquine and Quinine

Study Characteristics: Study Report 31, Part 2

Test Article:	Chloroquine Monodesethylchloroquine Didesethylchloroquine Quinine
Test System:	dog plasma
Internal Standard:	Neostigmine bromide
Sample Assay Volume:	200 µl
Sample Cleanup:	Precipitate proteins with methanol and acetonitrile

Analytical System

Detector:	MS/MS
Column Type:	Hypersil silica
Column Size:	4.6x50 mm, 3 µ particle size
Mobile Phase:	CH_3CN/H_2O (9:1, v/v) with 0.1% TFA and 5mM ammonium acetate

Validation Results: Chloroquine free base in dog plasma

Lower Limit of Quantitation:	4 ng/ml
Interday Mean, CV and RE:	3.69 ng/ml, 9.38%, and –7.67%
Intraday Mean, CV and RE:	3.78 ng/ml, 8.74%, and –5.42%
Standard curve range:	4.00 to 800 ng/ml
Interday Precision Concentrations:	8, 40, 160, and 600 ng/ml
CV Range:	7.36% to 11.0%
RE Range:	+2.13% to +5.52%
Intraday Precision Concentrations:	8, 40, 160, and 600 ng/ml
CV Range:	2.83% to 6.44%
RE Range:	+0.139% to +8.75%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	84.3%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	4 hours at ambient temp. 4°C for 16 days
Plasma Storage:	6 hours at ambient temp.
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	Not determined

Validation Results: Monodesethylchloroquine free base in dog plasma

Lower Limit of Quantitation:	4 ng/ml
Interday Mean, CV and RE:	3.50 ng/ml, 3.83%, and –12.4%
Intraday Mean, CV and RE:	3.59 ng/ml, 9.85%, and –10.3%
Standard curve range:	4.00 to 800 ng/m
Interday Precision Concentrations:	8, 40, 160, and 600 ng/ml
CV Range:	4.61% to 12.8%
RE Range:	+0.729% to +8.85%
Intraday Precision Concentrations:	8, 40, 160, and 600 ng/ml
CV Range:	3.01% to 6.79%
RE Range:	+0.083% to +11.6%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	79.9%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	4 hours at ambient temp.
	4°C for 16 days
Plasma Storage:	6 hours at ambient temp.
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	Not determined

Validation Results: Didesethylchloroquine free base in dog plasma

Lower Limit of Quantitation:	4 ng/ml
Interday Mean, CV and RE:	4.29 ng/ml, 7.45%, and +7.17%
Intraday Mean, CV and RE:	3.99 ng/ml, 16.1%, and –0.167%
Standard curve range:	4.00 to 800 ng/m
Interday Precision Concentrations:	8, 40, 160, and 600 ng/ml
CV Range:	6.09% to 12.1%
RE Range:	+0.771% to +4.10%
Intraday Precision Concentrations:	8, 40, 160, and 600 ng/ml
CV Range:	1.92% to 5.46%
RE Range:	+0.944% to +11.4%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	77.4%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	4 hours at ambient temp.
	4°C for 16 days
Plasma Storage:	6 hours at ambient temp.
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	Not determined

Validation Results: Quinine free base in dog plasma

Lower Limit of Quantitation:	10 ng/ml
Interday Mean, CV and RE:	10.6 ng/ml, 5.81%, and +6.27
Intraday Mean, CV and RE:	10.6 ng/ml, 6.69%, and +5.97
Standard curve range:	10 to 2000 ng/ml
Interday Precision Concentrations:	20, 100, 400, and 1500 ng/ml
CV Range:	7.71% to 10.7%
RE Range:	–0.067% to +3.44%
Intraday Precision Concentrations:	20, 100, 400, and 1500 ng/ml
CV Range:	2.45% to 10.5%
RE Range:	+0556% to +4.33%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	80.2%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	4 hours at ambient temp.
	4°C for 16 days
Plasma Storage:	6 hours at ambient temp.
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	Not determined

Draft Study Report 31, Part II, on validation of chloroquine (and its metabolites) and quinine was submitted for review on January 13, 1999. In response to the review received February 4, 1999. the final Study Report 31, Part II, was submitted May 13, 1999.

Study Description: This report describes the analytical method and validation of the analytical method used to measure concentrations of chloroquine (and its metabolites) and quinine in dog plasma samples. The method was developed and validated at the Analytical Division, Drug Studies Unit (DSU), UCSF, San Francisco, CA. The method was requested by the Contracting Officer's Representative at the Walter Reed Army Institute of Research (WRAIR) for contract DAMD17-97-C-7058 for validation of a method for a study of WR 238,605 used in combination with mefloquine, chloroquine, quinine, halofantrine and doxycycline in dog plasma and subsequent studies.

Method Summary: Dog plasma samples (200 µl) were analyzed for chloroquine (and its metabolites) and quinine with an LC/MS/MS procedure in a PE Sciex-API III® triple quadrupole system that uses a silica gel column, an acetonitrile/water/TFA (90:10:0.1, v/v) with 5 mM ammonium acetate mobile phase, and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (Atmospheric Pressure Chemical Ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample cleanup consisted of addition of methanol, precipitation with a neostigmine

internal standard solution in acetonitrile, centrifugation and transfer of the supernatant prior to separation by LC/MS/MS.

Validation Results Summary: The following validation parameters were evaluated for chloroquine (CHL) (and its metabolites didesethylchloroquine (DCL), monodesethylchloroquine (MCL)) and quinine (QUI).

1. Specificity: No significant endogenous interfering peaks for chloroquine (or its metabolites), quinine or for the internal standard were observed in blank dog plasma.

2. Inter-Day Precision and Accuracy: Interday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of dog plasma spiked with known amounts of drug. Each of 6 sets (n=2) of control samples at 4 different drug concentrations was evaluated (6 standard curves for the drug were run). Precision coefficients of variation (CV), ranged from 7.36% to 11.0% for CHL, 6.09% to 12.1% for DCL, 4.61% to 12.8% for MCL, and from 7.71% to 10.7% for QUI. The accuracy, defined by the relative error (RE) ranged from +2.13% to +5.52% for CHL, +0.771% to +4.10% for DCL, +0.729% to +8.85% for MCL and from –0.067% to +3.44% for QUI.

3. Intra-Day Precision and Accuracy: Intraday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of dog plasma spiked with known amounts of drug. For intraday precision, 6 sets (n=1) of control samples for each of four different drug concentrations were evaluated with one standard curve on the same run. CVs ranged from 2.83% to 6.44% for CHL, 1.92% to 5.46% for DCL, 3.01% to 6.79% for MCL, and from 2.45% to 10.5% for QUI. The REs ranged from +0.139% to +8.75% for CHL, +0.944% to +11.4% for DCL, +0.083% to +11.6% for MCL and from +0556% to +4.33% for QUI.

4. Lower Limit of Quantitation (LLOQ): The LLOQs for this assay are equivalent to the low points of the standard curves, or 4.00 ng/ml for CHL, DCL, and MCL and 10.0 ng/ml for QUI. Interday mean, CV, and RE results are 3.69 ng/ml, 9.38%, and –7.67% for CHL, 4.29 ng/ml, 7.45%, and +7.17% for DCL, and 3.50 ng/ml, 3.83%, and –12.4% for MCL and 10.6 ng/ml, 5.81%, and +6.27 for QUI. Intraday mean, CV, and RE results are 3.78 ng/ml, 8.74%, and –5.42% for CHL, 3.99 ng/ml, 16.1%, and –0.167% for DCL, and 3.59 ng/ml, 9.85%, and –10.3% for MCL and 10.6 ng/ml, 6.69%, and +5.97 for QUI.

5. Linear Range: The validated linear concentration ranges for this assay were 4.00 to 800 ng/ml for CHL, DCL, and MCL and 10.0 to 2000 ng/ml for QUI.

6. Recovery: Peak area ratios of processed samples to unprocessed samples provided overall recoveries of 84.3% for CHL, 77.4% for DCL, 79.9% for MCL and 80.2% for QUI.

7. Stability:

a. Freeze/Thaw: CHL, DCL, MCL and QUI were shown to be stable in dog plasma for up to 5 freeze/thaw cycles when samples are frozen to -70°C and thawed to room temperature.

b. Bench Top: CHL, DCL, MCL and QUI were shown to be stable for at least 6 hours in dog plasma at ambient temperature.

c. Processed Sample – Ambient temperature: CHL, DCL, MCL, QUI and internal standard were shown to be stable up to 4 hours at ambient temperatures.

Processed Sample - Refrigerated: CHL, DCL, MCL, QUI and internal standard were shown to be stable up to sixteen days at 4°C.

d. Long Term: A freezer (-70°C) storage stability test was not performed.

Method: Dog plasma samples (200 µl) were analyzed for chloroquine, monodesethyl-chloroquine, didesethylchloroquine, and quinine with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a silica column (4.6 x 50 mm, 3 µm particle size), 90% CH_3CN, 0.1% trifluoroacetic acid (TFA) and 5 mM ammonium acetate mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (atmospheric pressure chemical ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of 100 µl of methanol and of neostigmine internal standard (IS), mixing of the mixture, centrifugation and transfer of the supernatant prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free dog plasma samples with known amounts of chloroquine, monodesethyl-chloroquine, didesethylchloroquine, quinine, and IS. Standard curve, QC and assay samples were prepared as described, then ~8 µl aliquots were injected into the LC/MS/MS system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios of chloroquine (daughter ion at 247 *m/z* from parent ion at 321 *m/z*) and monodesethylchloroquine (daughter ion at 114 *m/z* from parent ion at 292 *m/z*), didesethylchloroquine (daughter ion at 179 *m/z* from parent ion at 265 *m/z*), and quinine (daughter ion at 251 *m/z* from parent ion at 325 *m/z*) to IS (daughter ion at 209 *m/z* from parent ion at 72 *m/z*) were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations of chloroquine, monodesethyl-chloroquine, didesethylchloroquine, and quinine to IS peak area ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the two equations for the best straight lines ($y = mx + b$, where y = peak area ratio and x = chloroquine, monodesethylchloroquine, didesethyl-chloroquine, or quinine concentrations), and drug and metabolite concentrations in assay samples were calculated by these equations from the

chloroquine, monodesethylchloroquine, didesethylchloroquine, and quinine to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank dog plasma with chloroquine, monodesethyl-chloroquine, didesethylchloroquine, and quinine.

Retention times (in minutes) were internal standard 1:12-14, chloroquine 1:39-40, monodesethylchloroquine 1:24-27, didesethylchloroquine1:25-28, and quinine1:03-09. The time between injections was 2-3 minutes.

Conclusion: The LC/MS/MS method for analysis of dog plasma to determine concentrations of chloroquine (and its metabolites) and quinine was validated for the concentration ranges of 4.00 ng/ml to 800 ng/ml for Chloroquine and Monodesethylchloroquine, and Didesethylchloroquine and 10.0 ng/ml to 2000 ng/ml for Quinine. "Preparation time for a run of 40 sponsor samples plus standard curve and control samples is about 1&1/2 hours. Run time is about 3&1/4 hours." The method was demonstrated to be precise, accurate, and sufficiently reproducible for analysis of study samples.

Study Report 31, Part III: Doxycycline

Study Characteristics: Study Report 31, Part 3

Test Article:	Doxycycline
Test System:	dog plasma
Internal Standard:	Minocycline
Sample Assay Volume:	200 µl
Sample Cleanup:	Precipitate proteins with methanol and acetonitrile

Analytical System

Detector:	MS/MS
Column Type:	Hypersil C8
Column Size:	4.6x50 mm, 3 µ particle size
Mobile Phase:	CH_3CN/H_2O (35:65, v/v) with 0.1% TFA and 0.01% dibasic ammonium phosphate

Validation Results: chloroquine free base in dog plasma

Lower Limit of Quantitation:	50 ng/ml
Interday Mean, CV and RE:	50.1 ng/ml, 8.74%, and +0.200%
Intraday Mean, CV and RE:	54.6 ng/ml, 11.3%, and +9.10%
Standard curve range:	50 to 12800 ng/ml
Interday Precision Concentrations:	100, 400, 1600 and 6400 ng/ml
CV Range:	5.93% to 10.5%
RE Range:	–3.33% to +0.833%
Intraday Precision Concentrations:	100, 400, 1600 and 6400 ng/ml
CV Range:	2.40% to 4.86%
RE Range:	+0.859% to +2.71%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	82.3%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	3 hours at ambient temp. 4°C for 16 days
Plasma Storage:	6 hours at ambient temp.
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	Not determined

Draft Study Report 31, Part III, on doxycycline was submitted for review on March 24 1999 and response to the review received June 3, 1999 is in preparation.

Study Description: This report describes the analytical method and validation of the analytical method used to measure concentrations of doxycycline in dog

plasma samples. The method was developed and validated at the Analytical Division, Drug Studies Unit (DSU), UCSF, San Francisco, CA.

Method Summary: Dog plasma samples (200 µl) were analyzed for Doxycycline (free base) with an LC/MS/MS procedure in a PE Sciex-API III® system that uses a C8 column, an acetonitrile/water/TFA/$(NH_4)_2HPO_4$ (35:65:0.1:0.01, v/v) mobile phase, and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (Atmospheric Pressure Chemical Ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample cleanup consisted of addition of methanol, precipitation with a minocycline internal standard solution in acetonitrile, centrifugation and transfer of the supernatant prior to separation by LC/MS/MS.

Validation Results Summary: The following validation parameters were evaluated for Doxycycline.

1. Specificity: No significant endogenous interfering peaks for doxycycline or for the internal standard were observed in blank dog plasma.

2. Inter-Day Precision and Accuracy: Interday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of dog plasma spiked with known amounts of drug. Each of 6 sets (n=2) of control samples at 4 different drug concentrations was evaluated (6 standard curves for the drug were run). Precision coefficients of variation (CV), ranged from 5.93% to 10.5% for doxycycline. The accuracy, defined by the relative error (RE) ranged from –3.33% to +0.833% for doxycycline.

3. Intra-Day Precision and Accuracy: Intraday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of dog plasma spiked with known amounts of drug. For intraday precision, 6 sets (n=1) of control samples for each of 4 different drug concentrations were evaluated with 1 standard curve on the same run. CVs ranged from 2.40% to 4.86% for doxycycline. REs ranged from +0.859% to +2.71% for doxycycline.

4. Lower Limit of Quantitation (LLOQ): The LLOQs for this assay are equivalent to the low points of the standard curves, or 50.0 ng/ml for doxycycline. Interday mean, CV, and RE results are 50.1 ng/ml, 8.74%, and +0.200% for doxycycline. Intraday mean, CV, and RE results are 54.6 ng/ml, 11.3%, and +9.10% for doxycycline.

5. Linear Range: The validated linear concentration range for this assay was 50.0 to 12800 ng/ml for doxycycline.

6. Recovery: Peak area ratios of processed samples to unprocessed samples provided an overall recovery of 82.3% for doxycycline.

7. Stability:

    a. Freeze/Thaw: Doxycycline was shown to be stable in dog plasma for up to 5 freeze/thaw cycles when samples are frozen to -70°C and thawed to room temperature.

b. Bench Top: Doxycycline was shown to be stable for at least 6 hours in dog plasma at ambient temperature.

c. Processed Sample – Ambient temperature: Doxycycline and internal standard were shown to be stable up to 3 hours at ambient temperatures.

Processed Sample - Refrigerated: Doxycycline and internal standard were shown to be stable up to 16 days at 4°C.

d. Long Term: A freezer (-70°C) storage stability test was not performed.

Method: Dog plasma samples (200 µl) were analyzed for doxycycline with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a C8 column (4.6 x 50 mm, 3 µm particle size) and 35% CH_3CN, 0.1% trifluoroacetic acid (TFA), and 0.01% ammonium phosphate mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (atmospheric pressure chemical ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of 100 µl of methanol, addition of minocycline internal standard (IS), and transfer of the supernatant prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free dog plasma samples with known amounts of doxycycline and IS. Standard curve, QC and assay samples were prepared as described, then injected into the LC/MS/MS system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios of doxycycline to IS were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations and doxycycline to IS peak area ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the two equations for the best straight lines ($y = mx + b$, where y = peak area ratio and x = doxycycline concentrations), and drug concentrations in assay samples were calculated by these equations from the doxycycline to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank dog plasma with doxycycline.

Conclusion: The LC/MS/MS method for analysis of dog plasma to determine concentrations of doxycycline was validated for the concentration ranges of 50.0 ng/ml to 12800 ng/ml for doxycycline. The method was demonstrated to be precise, accurate, and sufficiently reproducible for analysis of study samples.

Study Report 31, Part IV: Halofantrine, its Metabolite, and WR 238,605

Study Characteristics: Study Report 31, Part 4

Test Article:	Halofantrine WR 178,460 WR 238,608
Test System:	dog plasma
Internal Standard:	Verapamil
Sample Assay Volume:	100 µl
Sample Cleanup:	Precipitate proteins with methanol and acetonitrile

Analytical System

Detector:	MS/MS
Column Type:	Hypersil silica
Column Size:	4.6x50 mm, 3 µ particle size
Mobile Phase:	CH_3CN/H_2O (9:1, v/v) with 0.06%TFA

Validation Results: Halofantrine free base in dog plasma

Lower Limit of Quantitation:	2 ng/ml
Interday Mean, CV and RE:	1.62 ng/ml, 5.27%, and -19.0%
Intraday Mean, CV and RE:	1.73 ng/ml, 9.84%, and -13.4%
Standard curve range:	2.00 to 400 ng/ml
Interday Precision Concentrations:	4, 20, 80, and 320 ng/ml
CV Range:	2.89% to 8.86%
RE Range:	-6.25% to +8.17%
Intraday Precision Concentrations:	4, 20, 80, and 320 ng/ml
CV Range:	2.75% to 6.32%
RE Range:	-1.73% to +7.08%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	82.1%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	Not determined
Plasma Storage:	3 days at ambient temp.
5 Cycle Freeze/Thaw:	Not determined
Standard Solution:	Not determined

Validation Results: WR 178,460 free base in dog plasma

Lower Limit of Quantitation:	2 ng/ml
Interday Mean, CV and RE:	1.74 ng/ml, 8.92%, and -13.0%
Intraday Mean, CV and RE:	1.76 ng/ml, 17.5%, and -11.9%
Standard curve range:	2.00 to 400 ng/ml
Interday Precision Concentrations:	4, 20, 80, and 320 ng/ml
CV Range:	2.35% to 8.51%
RE Range:	-6.29% to +8.00%
Intraday Precision Concentrations:	4, 20, 80, and 320 ng/ml
CV Range:	2.62% to 7.69%
RE Range:	-5.21% to +4.83%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	78.9%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	Not determined
Plasma Storage:	3 days at ambient temp.
5 Cycle Freeze/Thaw:	Not determined
Standard Solution:	Not determined

Validation Results: WR 238,608 free base in dog plasma

Lower Limit of Quantitation:	2 ng/ml
Interday Mean, CV and RE:	2.02 ng/ml, 7.76%, and +1.17%
Intraday Mean, CV and RE:	2.18 ng/ml, 1.86%, and +9.00%
Standard curve range:	2.00 to 400 ng/ml
Interday Precision Concentrations:	4, 20, 80, and 320 ng/ml
CV Range:	3.73% to 8.93%
RE Range:	-3.08% to +6.25%
Intraday Precision Concentrations:	4, 20, 80, and 320 ng/ml
CV Range:	3.05% to 10.9%
RE Range:	-3.21% to +7.08%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	82.0%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	Not determined
Plasma Storage:	3 days at ambient temp.
5 Cycle Freeze/Thaw:	Not determined
Standard Solution:	Not determined

Draft Study Report 31, Part IV, on halofantrine and WR 238605 was submitted for review on April 22 1999 and response to the review received June 3, 1999 is in preparation.

Study Description: This report describes the analytical method and validation of the analytical method used to measure concentrations of Halofantrine, Halofantrine metabolite and WR 238,605 in dog plasma samples. The method was developed and validated at the Analytical Division, Drug Studies Unit (DSU), UCSF, San Francisco, CA.

Method Summary: Dog plasma samples (100 µl) were analyzed for Halofantrine, Halofantrine metabolite and WR 238,605 (as free bases) with an LC/MS/MS procedure in a PE Sciex-API III® system that uses a silica gel column, an acetonitrile/water/TFA (90:10:0.06, v/v) mobile phase, and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (Atmospheric Pressure Chemical Ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample cleanup consisted of addition of methanol, precipitation with a verapamil internal standard solution in acetonitrile, centrifugation and transfer of the supernatant prior to separation by LC/MS/MS.

Validation Results Summary: The following validation parameters were evaluated for Halofantrine, Halofantrine metabolite and WR 238,605.

1. Specificity: No significant endogenous interfering peaks for Halofantrine, Halofantrine metabolite, WR 238,605 or for the internal standards were observed in blank dog plasma.

2. Inter-Day Precision and Accuracy: Interday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of dog plasma spiked with known amounts of drug. Each of 3 sets (n=2) of control samples at 4 different drug concentrations was evaluated (3 standard curves for the drug were run). Precision coefficients of variation (CV), ranged from 2.89% to 8.86% for Halofantrine, from 2.35% to 8.51% for Halofantrine metabolite and from 3.73% to 8.93% for WR 238,605. The accuracy, defined by the relative error (RE) ranged from -6.25% to +8.17% for Halofantrine, from -6.29% to +8.00% for Halofantrine metabolite and from -3.08% to +6.25% for WR 238,605.

3. Intra-Day Precision and Accuracy: Intraday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of dog plasma spiked with known amounts of drug. For intraday precision, 6 sets (n=1) of control samples for each of 4 different drug concentrations were evaluated with 1 standard curve on the same run. CVs ranged from 2.75% to 6.32% for Halofantrine, from 2.62% to 7.69% for Halofantrine metabolite and from 3.05% to 10.9% for WR 238,605. REs ranged from -1.73% to +7.08% for Halofantrine, from -5.21% to +4.83% for Halofantrine metabolite and from -3.21% to +7.08% for WR 238,605.

4. Lower Limit of Quantitation (LLOQ): The LLOQs for this assay are equivalent to the low points of the standard curves, or 2.00 ng/ml for Halofantrine, Halofantrine metabolite and WR 238,605. Interday mean, CV, and RE results are 1.62 ng/ml, 5.27%, and -19.0% for Halofantrine, 1.74 ng/ml, 8.92%, and -13.0% for Halofantrine metabolite and 2.02 ng/ml, 7.76%, and +1.17% for WR 238,605. Intraday mean, CV, and RE results are 1.73 ng/ml, 9.84%, and -13.4% for Halofantrine, 1.76 ng/ml, 17.5%, and -11.9% for Halofantrine metabolite and 2.18 ng/ml, 1.86%, and +9.00% for WR 238,605.

5. Linear Range: The validated linear concentration ranges for this assay were 2.00 to 400 ng/ml for Halofantrine, Halofantrine metabolite and WR 238,605.

6. Recovery: Peak area ratios of processed samples to unprocessed samples provided overall recoveries of 82.1% for Halofantrine, 78.9% for Halofantrine metabolite and 82.0% WR 238,605.

7. Stability:

   a. Bench Top: Halofantrine, Halofantrine metabolite and WR 238,605 were shown to be stable for at least 3 days in dog plasma at ambient temperature.

   b. Freeze/Thaw, Processed Sample – Ambient temperature, Processed Sample – Refrigerated, Long Term freezer storage stability tests were not performed for this study.

Method: Dog plasma samples (100 µl) were analyzed for Halofantrine, Halofantrine metabolite and WR 238,605 (Halofantrine and metabolite were analyzed in a separate run from WR 238,605) with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a silica column (4.6 x 50 mm, 3 µm particle size), 90% CH_3CN, 0.06% trifluoroacetic acid (TFA) mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (atmospheric pressure chemical ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of 100 µl of methanol, addition of verapamil/WR 122455 internal standard (IS) in acetonitrile, and transfer of the supernatant prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free dog plasma samples with known amounts of Halofantrine, Halofantrine metabolite, WR 238,605, and IS. Standard curve, QC and assay samples were prepared as described, then 3-5 µl aliquots were injected into the LC/MS/MS system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios of Halofantrine, Halofantrine metabolite and WR 238,605 to IS were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations and Halofantrine, Halofantrine metabolite and WR 238,605 to IS peak area ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the equations for the best straight lines ($y = mx + b$,

where y = peak area ratio and x = Halofantrine, Halofantrine metabolite or WR 238,605 concentrations), and drug concentrations in assay samples were calculated by these equations from the Halofantrine, Halofantrine metabolite and WR 238,605 to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank dog plasma with Halofantrine, Halofantrine metabolite and WR 238,605.

Conclusion: The LC/MS/MS method for analysis of dog plasma to determine concentrations of Halofantrine, Halofantrine metabolite and WR 238,605 was validated for the concentration ranges of 2.00 ng/ml to 400 ng/ml for Halofantrine, Halofantrine metabolite and WR 238,605. The method was demonstrated to be precise, accurate, and sufficiently reproducible for analysis of study samples.

Study Report 32: WR 238,608 in Human Plasma

Study Characteristics: Study Report 32

Test Article:	WR 238,608
Test System:	human plasma
Internal Standard:	Verapamil
Sample Assay Volume:	100 µl
Sample Cleanup:	acetonitrile precipitation

Analytical System

Detector:	MS/MS
Column Type:	hypersil silica
Column Size:	4.6x50 mm, 5µ particle size
Mobile Phase:	acetonitrile/water/TFA (90:10:0.06, v/v/v).

Validation of a method for WR 238,605 in human plasma and blood by LC/MS/MS has been completed and a report is in preparation. A long term stability study for WR 238,605 in human blood and plasma (plasma data to 21 months and blood to 7 months was Faxed to the COR on May 22, 1998) for up to two years requested in a COR letter dated Feb. 15, 1996 continued. Results on analyses of 35 blind human plasma samples received February 25, 1998 from WRAIR were Faxed to the COR on May 21, 1998.

A small volume sample (50 µl blood) method described in Faxes from the COR dated April 13, 21 and 24, 1998 has been validated and a report is in preparation.

Study Report 33: Halofantrine and WR 178460 in Human Plasma and Blood

Study Characteristics: Study Report 33

Test Article:	Halofantrine WR 178460
Test System:	human plasma and blood
Analytical System	
Detector:	MS/MS

Long term (2 year) freezer stability study and LC/MS/MS method development is in progress for halofantrine and desbutylhalofantrine in human plasma and blood.

Study Report 34: WR 254421 in Human Plasma

Study Characteristics: Study Report 34

Test Article:	WR 254421
Test System:	human plasma
Analytical System	
Detector:	MS/MS

This project was requested as described in a COR fax dated November 12, 1997 for quantitation of WR 254,421 (as Free Base) in human plasma. Assay development is in progress.

Study Report 35: Artelinic Acid in Rat and Dog Plasma

Study Characteristics: Study Report 35	
Test Article:	Artelinic acid
Test System:	rat plasma
Analytical System	
Detector:	MS/MS

LC/MS/MS method development is in progress for artelinic acid in rat and dog plasma. This project was described in a COR fax dated July 22, 1998.

Routine Assay Results

The following section presents short descriptions of specific routine sample assays completed or currently in progress during the contract. Complete annual data findings are presented in Appendix B.

TABLE 5: CURRENT ROUTINE ANALYSES

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine analysis for Halofantrine and WR 178,460 (as f.b.) of Plasma Samples Obtained for the Protocol Titled "Pharmacokinetics of a New Multiple Dose Halofantrine Regimen"	12/10/96 draft in review by COR	Halofantrine WR 178,460	human plasma	642 642	Hal/P 93-2
No protocol	2/25/94 data, draft in preparation	*p*-aminoheptanophenone	dog plasma	876	Pah/P 93-3
Routine Analysis for WR 238,605 (as f.b.) of Plasma Samples Obtained for the Protocol Titled "Thirteen Week Oral Toxicity Study of WR 238,605 with a Thirteen Week Recovery Period in Dogs"	9/16/98 final report	WR 238,605	dog plasma	330	WR5/P 93-4
Routine Analysis for Halofantrine and WR 178,460 (as f.b.) of Rat Liver, Bile and Perfusate Samples	10/28/94 final data, draft report in preparation	halofantrine	rat liver perfsate bile	no count	Hal/lpb 93-7
Routine Analysis for WR 238,605 (as f.b.) Human Plasma and Blood Samples Obtained for the Protocol Titled "Pharmacokinetics, Pharmacodynamics, Safety and Tolerance of a Single Oral Dose of WR 238605 Succinate"	2/7/95 final data, draft report in preparation	WR 238,605	human plasma blood	359 359	WR5/PB 93-8

TABLE 5: CURRENT ROUTINE ANALYSES

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis for *p*-Aminoheptanophenone of Dog Plasma Samples Obtained for the Protocol Titled "*p*-Aminoheptanophenone (PAHP) (WR269410) Single Dose IV and Oral Pharmacokinetic, Pharmacodynamic, Bioavailability and Metabolism Study in Dogs"	2/7/95 final data, draft report in preparation	*p*-aminoheptanophenone	dog plasma	189	Pah/P 93-9
Routine Analysis for WR 238,605 (as free base)Monkey Plasma Samples	11/22/94 final data, letter report in preparation	WR 238,605	monkey plasma	12	WR5/P 94-1
Routine Analysis for *p*-Aminoheptanophenone Rat Plasma Samples Obtained for the Protocol Titled "p-Aminoheptanophenone (PAHP) (WR269410) Single Dose IV and Oral Pharmacokinetic, Pharmacodynamic, Bioavailability and Metabolism Study in Rats"	2/7/95 final data, draft in preparation	*p*-aminoheptanophenone	rat plasma	152	Pah/P 94-2
Tentative Title: Routine Analysis for WR 6026 and Metabolites in Plasma and Urine Samples Obtained for the Protocol Titled "Clinical Trial of Oral WR6026•2HCl in Patients with Brazilian Visceral Leishmaniasis due to L. chagasi: Initial Dose Range Determine	8/22/97 final data final data in progress final data final data final data	 WR 6026 WR 211789 WR 254421 WR 6026 WR 211789 WR 254421	human plasma urine	120 90	WR6/PU 94-3
Tentative Title: Routine Analysis for WR 238605 in Plasma Samples Obtained for the Protocol Titled "Evaluation of WR 238605 as a Prophylactic Agent against Induced P. falciparum Malaria Infection in Healthy Non-immune Subjects: A Dose Ranging Study"	11/21/94 final data, draft report in preparation	WR 238,605	human plasma blood	28 28	WR5/PB 94-4

TABLE 5: CURRENT ROUTINE ANALYSES

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis for WR 238605 in Plasma Samples Obtained for the Protocol Titled "A Multiple Dose Safety, Tolerance and Pharmacokinetic Study of WR 238605 when Given to Healthy Male and Female Subjects"	8/27/98 final report	WR 238605	human plasma	709	WR5/P 94-7
Routine Analysis for WR 238,605 (f.b.) of Human Plasma and Blood Samples and for Chloroquine and Chloroquine Metabolite of Human Blood Samples Obtained for the Protocol Titled "Evaluation of WR 238605 as a Prophylactic Agent against Induced *P. falciparum* Malaria Infection in Healthy Non-Immune Subjects II: A Multiple-Dose Causal vs. Suppressive Study"	8/28/98 final report	WR 238605 chloroquine monodesethyl chloroquine	human plasma blood blood blood	226 226 67 67	WR5/P 95-2
Routine Analysis for the R andS Isomers of WR 238,605 (f.b.) of Human Plasma Samples Obtained for the Protocol Titled "Evaluation of WR 238605 as a Prophylactic Agent against Induced *P. falciparum* Malaria Infection in Healthy Non-Immune Subjects II: A Multiple-Dose Causal versus Suppressive Study"	9/2/98 final report	*R* WR 238605 *S* WR 238605	human plasma	226 226	WR5/P 95-2
Routine Analysis for WR 238605 in Plasma Samples Obtained for the Protocol Titled "WR 238605 Multiple Drug Interaction Study in Beagle Dogs"	6/11/99 draft report in review by COR	WR 238605 Mefloquine Chloroquine Quinine Doxycycline Halofantrine	human plasma	1084	WR5/P 95-3

TABLE 5: CURRENT ROUTINE ANALYSES

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis for Halofantrine and WR 178460 in Plasma Samples Obtained for the Protocol Titled "Halofantrine as Prophylaxis against Malaria: Multiple-Dose Safety, Tolerance and Pharmacokinetics Study"	11/24/97 final data chiral 5/4/98 final data Draft report in preparation	Halofantrine WR 178,460 Halofantrine WR 178,460	human plasma plasma	1365 1365 1365 1365	Hal/P 95-4
Tentative Title: Routine Analysis for WR 238605 in Plasma Samples Obtained for the Protocol Titled "Dose-Ranging Study of the Safety and Efficacy of WR 238605 in the Prevention of Relapse of Plasmodium vivax Infection in Thailand"	3/2/98 final data submitted	WR238605 Chloroquine	human plasma blood plasma blood	558 552 558 552	WR5/BP 96-2
Routine Analysis for Gentamicin/Paromomycin (as Free Bases) of Human Plasma Samples Obtained for the Protocol Titled "Irritant and Phototoxicity Reactions to the Topical Antileischmanial WR 279,369: A Randomized, Double-Blind Phase I Study"	3/12/98 draft report in revision	Gentamicin Paromomycin	human plasma	36 47	Gnt/P 96-3
Routine Analysis for WR238605 in Dog Plasma Samples for the Protocol Titled "One Year Oral Toxicity Study of WR 238605"	1/5/99 draft report in revision	WR238605	dog plasma	224	WR5/P 97-1
Routine Analysis for Mefloquine Chloroquine and Primaquine in Plasma Samples	12/11/97 final data, draft report in preparation	Mefloquine Chloroquine Primaquine	plasma	14 2 2	MEF/P 97-2

p-Aminoheptanophenone (WR 269,410), WR 258,948 and WR 302

Pah/P 93-3
(analytical data was presented in the DAMD17-92-C-2028 mid-term report)

Results will be reported in Analysis Report No. 93-3. Status of samples received is described in the table below. Report completion requires completion of method validation.

No. of Samples	Description	Date Received	Status
106	dog plasma	3/3/93	Results Faxed to COR 9/23/93
52	dog blood	3/3/93	Not to be assayed
645	dog plasma	9/21/93	Results Faxed to COR 2/25/94
36	blind spiked dog plasma	9/30/93 11/2/93	Results Faxed to COR 1/25/94
125	dog plasma	10/21/93	Results Faxed to COR 2/25/94

Pah/P 93-9
(analytical data was presented in the DAMD17-92-C-2028 final report)

Samples (189 dog plasma) were received July 12, 1994 to be analyzed in accordance with the protocol titled "*p*-Aminoheptanophenone (PAHP) (WR269410) Single Dose IV and Oral Pharmacokinetic, Pharmacodynamic, Bioavailability and Metabolism Study in Dogs." Analysis is complete and final results were Faxed to the COR on February 7, 1995. Report completion requires completion of the method validation report.

Pah/P 94-2
(analytical data was presented in the DAMD17-92-C-2028 final report)

Samples (152 rat plasma) were received July 12, 1994 to be analyzed in accordance with the protocol titled "*p*-Aminoheptanophenone (PAHP) (WR269410) Single Dose IV and Oral Pharmacokinetic, Pharmacodynamic, Bioavailability and Metabolism Study in Rats." Analysis is complete and final results were Faxed to the COR on February 7, 1995. Report completion requires completion of the method validation report.

HALOFANTRINE

Hal/P 93-2
(analytical data was presented in the DAMD17-92-C-2028 final report)

Analysis of 642 human plasma samples for determination of the free base concentrations of halofantrine (WR 171,669) and of its metabolite (WR 178,460) was accomplished by use of an HPLC method described in Study Report 17, developed under contract DAMD17-86-C-6150. The samples were obtained from the South Florida Drug Research Corporation, Inc., in accordance with the protocol titled "Pharmacokinetics of a New Multiple Dose Halofantrine

Regimen." Analytical results were presented in Analysis Report Hal/P 93-2 submitted for review on December 10, 1996, for plasma samples from human male subjects from analyses performed from April 30 through June 8, 1993.

Hal/lpb 93-7
(analytical data was presented in the DAMD17-92-C-2028 final report)

Final bile, liver and perfusate results were attached to Quarterly Report 11. Additional data, showing just perfusate extraction results, were Faxed December 28, 1994. Analysis Report Hal/Lprb 93-7 is in preparation. Remaining samples were returned to WRAIR on July 25, 1995.

Hal/P 95-4

Samples are to be analyzed in accordance to the protocol titled "Halofantrine as Prophylaxis against Malaria: Multiple-Dose Safety, Tolerance and Pharmacokinetics Study." Final data on 1060 samples for halofantrine and WR 178,460 (free base) concentrations were Faxed to the COR on January 3, 1997. Final results on 305 samples received on June 10, 1997 were Faxed to the COR on November 24, 1997. Results on the chiral assay of the same samples were Faxed to the COR on May 4, 1998. Analysis Report Hal/BP 95-4 is in preparation.

MEFLOQUINE

Mef/P 97-2

Analysis of 14 human plasma samples for determination of the free base concentration of mefloquine hydrochloride (WR 142,490), was accomplished by use of the HPLC method described in Study Report 14B dated August 29, 1989 under contract DAMD17-86-C-6150. Final mefloquine concentrations on 14 of 15 human plasma samples (15th=NS) were submitted in a fax dated November 13, 1997. Final primaquine, chloroquine and metabolite concentrations on 2 human plasma samples were submitted in a fax dated December 11, 1997. The samples were obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research. A report is in preparation.

WR 238,605

WR5/P 93-4

Results of the analysis of 330 dog plasma samples received on July 14, 1993 were reported in Routine Analysis Report WR5/P 93-4, which was submitted for review on April 25, 1994. The protocol for this study is titled "Thirteen week oral toxicity study of WR 238605 with a thirteen week recovery period in dogs." Study Report 13B, Supplement II for dog plasma validation, modified as requested in a COR letter dated January 31, 1995, mandates changes in this report. Sample results were discussed in an April 3, 1995 site visit meeting and in correspondence from the COR dated April 22, 1994 and May 26, 1994. No

further changes were required by the COR (July 27, 1998 fax) to finalize this report, and the final report was submitted September 16, 1998.

WR5/P 93-8
(analytical data was presented in the DAMD17-92-C-2028 final report)

Routine analysis of 359 human plasma and 359 human blood samples was completed for Analysis Report WR5/BP 93-8 for samples received in accordance with the protocol titled "Pharmacokinetics, Pharmacodynamics, Safety and Tolerance of a Single Oral Dose of WR 238605 Succinate." Final data was attached to Quarterly Report 11. Repeat analysis of selected samples, as requested in a FAX from the COR dated December 9, 1994, was completed and results were faxed to the COR February 7, 1995. A report is in preparation.

WR5/P 94-1
(analytical data was presented in the DAMD17-92-C-2028 final report)

Monkey blood (12) samples were received September 15, 1994. Final analytical results were faxed to the COR on 11/22/94. A brief letter reporting results and referring to the human validation report as suggested by the COR at the April 3, 1995 site visit is in preparation. The analysis was set to proceed with use of blank human plasma for standard curve and control samples and blank monkey plasma as duplicate controls.

WR5/P 94-4
(analytical data was presented in the DAMD17-92-C-2028 final report)

Human plasma (28) and human blood (28) samples were received October 26, 1994 and assayed in accordance with the protocol titled "Evaluation of WR 238605 as a Prophylactic Agent against Induced *P. falciparum* Malaria Infection in Healthy Non-immune Subjects: A Dose Ranging Study." Final analytical results were faxed to the COR on 11/21/94 and the report is in preparation.

WR5/P 95-3
(analytical data is presented in Appendix A)

Dog plasma samples were analyzed in accordance to the protocol titled "WR 238605 Multiple Drug Interaction Study in Beagle Dogs" as requested in a COR letter dated October 20, 1995. Results on 131 samples (and 18 dosing solutions not reported) received April 25 and November 25, 1996 for WR 238,605, mefloquine, chloroquine, monodesethyl-chloroquine, didesethyl-chloroquine, quinine, doxycycline, halofantrine and/or WR 178460 were faxed to the COR on March 20, 1997. On January 13-14, 1998 an additional 431 plasma samples and 21 vials of dosing solutions were received. On February 10, 1998 an additional 522 plasma samples and 6 vials of dosing solutions were received. Final results were e-mailed to the COR on December 30, 1998. A draft report was submitted for review on June 11, 1999.

WR5/P 96-2

Samples are to be analyzed as requested in a COR letter dated May 6, 1996 in accordance with the protocol titled "Dose-Ranging Study of the Safety and Efficacy of WR 238605 in the Prevention of Relapse of *Plasmodium vivax* Infection in Thailand." Final results on 266 human plasma and 260 human blood samples for WR 238,605 were faxed to the COR on March 6, 1997. On December 17, 1997, an additional 357 human plasma and 358 human blood samples were received (a recount shows that 1 sample less was received than originally believed). On March 2, 1998, final WR 238,605 results on 292 plasma and 292 blood (excluding samples from subjects dosed only with chloroquine) were faxed to the COR. Chloroquine blood and plasma analyses have been run and data is being evaluated. Additional samples are expected (COR fax dated March 5, 1998).

WR5/P 97-1

Samples were to be analyzed in accordance with the protocol titled " One Year Oral Toxicity Study of WR 238605 Succinate in Dogs." Samples (224 dog plasma) were received August 13, 1997. Final data was faxed to the COR September 26, 1997 and a draft report was submitted for review January 5, 1999 that was found acceptable as described in a COR Fax dated January 20, 1999. A signed report will be submitted with the final validation report.

WR 6026, WR 211,789 and WR 254,421

WR6/PU 94-3

Samples were received for routine analysis in accordance with the protocol titled "Clinical Trial of Oral WR6026•2HCl in Patients with Brazilian Visceral Leishmaniasis due to *L. chagasi*: Initial Dose Range Determination for Efficacy, Safety and Tolerance." Final data on 92 human plasma and 90 human urine samples was Faxed to the COR on January 27, 1997 and on 28 human plasma samples was Faxed to the COR on August 22, 1997. On November 13, 1997, 92 human sera samples were received and a request for analysis of previously received plasma/sera samples was made for WR 254421 determinations.

GENTAMICIN AND PAROMOMYCIN

Gnt/p 96-3

Samples were analyzed in accordance with the protocol titled " Irritant and Phototoxicity Reactions to the Topical Antileishmanial WR 279396: A Randomized, Double-Blind Phase I Study." Final data on 83 human plasma samples was Faxed to the COR on December 18, 1996. Draft Analysis Report Gen/P 96-3 was submitted to the COR for review on March 12, 1998 and a revised report responding to changes requested in a fax dated June 14, 1999 is in preparation.

KEY RESEARCH ACCOMPLISHMENTS

Methods developed for:

- WR 238605 and mefloquine in dog plasma (SR31, part I)
- Chloroquine, its two metabolites, and quinine in dog plasma (SR31, part II)
- Doxycycline in dog plasma (SR31, part III)
- Halofantrine, its metabolite, and WR 238,605 in dog plasma (SR31, part IV)

Sample results for:

- 1084 results for WR 238605, mefloquine, chloroquine (and its two metabolites), quinine, doxycycline and halofantrine (and its metabolite) (in draft report WR5/P 95-3)

REPORTABLE OUTCOMES

Yeping Zhao applied for and received employment at Sugen Pharmaceuticals, Inc., and Keshi Wang applied for and received employment at Chiron, Inc., based in part on LC/MS/MS experience supported by this contract.

CONCLUSIONS

Using the procedures described in this report, we were able to work sequentially or simultaneously on development, validation and characterization of assays for WR 6026 (and its metabolites, WR 211789 and WR 254421), mefloquine (and its metabolite, WR 160972), *p*-aminoheptanophenone (and related compounds), WR 242511, halofantrine (and its metabolite, WR 178,460, and their stereoisomers), chloroquine (and its metabolites, monodesethylchloroquine and didesethylchloroquine), WR 243,251, WR 238,605, quinine, doxycycline, and artelinic acid. Work on routine analyses of biological specimens during this period was performed for studies that required determination of concentrations of WR 238,605, mefloquine, chloroquine (and its metabolites, monodesethylchloroquine and didesethylchloroquine), doxycycline, halofantrine (and its metabolite, WR 178,460), and quinine. We worked on demonstrating sensitivity, specificity, linearity, lack of interferences, accuracy, and reproducibility of the analytical method, describing the extent of recovery for the method, and reporting on the stability of compounds of interest in specimens during storage and drug analysis to provide documentation in support of Investigational New Drug (IND) submissions to the Food and Drug Administration (FDA).

REFERENCES

[1] Anticholinesterase Agents. Palmer Taylor in *"The Pharmacological Basis of Therapeutics"* (Editors) A.G. Gilman, L.S. Goodman, T.W. Rall and F. Murad. Seventh Edition, MacMillan Publishing Co. Chapter 6, pp. 118-129 (1985).

[2] Ion-Paired Liquid Chromatographic Method for the Analysis of Blood and Plasma for the Antimalarial Drug Halofantrine and its Putative Mono-debutylated Metabolite. M. Gawienowski, L.Z. Benet, L. Fleckenstein and E.T. Lin. *J. Liq. Chromatogr.* **30**, 412-419 (1988).

[3] Analysis of the Antileishmanial Compound WR 6026 by High Performance Liquid Chromatography. C.M. Chen, W.L. Gee, L.Z. Benet, J. von Bredow and L. Fleckenstein. *APhA Academy of Pharmaceutical Science* **14**, 216 (1984).

[4] Analysis of Artesunic Acid and Dihydroqinghaosu in Blood by High-Performance Liquid Chromatography with Reductiove Electrochemical Detection. Z.M. Zhou, J.C. Anders, H. Chung and A.D. Theoharides. *J. Chromatogr.* **414,** 77-90 (1987).

[5] Separation of Isomeric Insect Pheromonal Compounds Using Reversed Phase HPLC with $AgNO_3$ in the Mobil Phase. P.L. Phelan and J.R. Miller. *J. Chromatogr. Sci.* **19,** 13-17 (1981).

[6] *Organic Functional Group Analysis by Micro and Semimicro Methods.* N.D. Cheronis and T.S. Ma. Interscience, New York (1964).

[7] *Protective Groups in Organic Chemistry.* J.F.W. McOmie. Plenum, New York (1973).

[8] Ion-Paired Liquid Chromatographic Method for the Analysis of Pyridostigmine in Plasma. O. Yturralde, R-Y. Lee, L.Z. Benet, L. Fleckenstein, and E.T. Lin. *J. Liq. Chromatogr.* **10**, 2231-2246 (1987).

[9] Kinnamon, K.E.; Steck, E.A.; Loizeaux, P.S.; Hanson, W.L.; Chapman, W.L.; and Waits, V.B. *Amer, J. Trop. Med. Hyg.*, 27(4):751 (1978).

[10] Locksley, R.M. and Plorde, J.J.: "Leishmaniasis," in Harrison's Principles of Internal Medicine, Tenth edition. Edited by Petersdorf, R.G.; Adams, R.P.; Braunwald, E.; Isselbacher K.J.; Martin, J.B.; and Wilson, J.D. McGraw-Hill Book Co., New York, pp. 1193-6, 1983.

[11] Physicians' Desk Reference, 40th Edition. Publisher; Edward R. Barnhart, pp. 1073 and 1744-5.

[12] Lin, E.T.; Benet, L.Z.; Upton, R.A.; and Gee, W.L. Annual Report "Development of Analytical Methods for the Determination of Drug Substances in Biological Fluids," US Army Medical Research and Development Command Contract No. DAMD17-83-C-3004, August 26, 1983.

[13] Bidlingmeyer, B.A.; Del Rios, J.K.; and Korpi, J. Separation of Organic Amine Compounds on Silica Gel with Reversed-Phase Eluents. *Anal. Chem.* 54:442 (1982).

[14] Theoharides, A.D.; Chung, H.; and Velazquez, H. Metabolism of a Potential 8-Aminoquinoline Antileishmanial Drug in Rat Liver Microsomes," *Biochem. Pharmacol.* 34:181 (1985).

[15] Shi, R.J.Y.; Benet, L.Z.; and Lin, E.T. High Performance Liquid Chromatographic Assay of Basic Amine Drugs in Plasma and Urine Using a Silica Gel Column and an Aqueous Mobile Phase. *J. Chromatogr.* 377:399-404 (1986).

[16] Shi, J.Y.R.; Gee, W.L.; Williams, R.L.; and Lin, E.T. High Performance Liquid Chromatographic Assay of Metoclopramide in Plasma Using a Silica Gel Column and an Aqueous Mobile Phase. *Anal. Lett.* 20:131 (1987).

[17] Shi, J.Y.R.; Gee, W.L.; Williams, R.L.; and Lin, E.T. High Performance Liquid Chromatographic Assay for Basic Amine Drugs in Plasma and Urine Using a Silica Gel Column and an Aqueous Mobile Phase. II. Chloropheniramine. *J. Liq. Chromatogr.* 10(4), 3101-3112 (1987).

[18] C.J. Canfield and R. S. Rozman, *Bull. WHO*, 50 (1974) 203.

[19] G.M. Trenholme, R. L. Williams, R. Z. Desjardins, H. Frischer, P. E. Carson, K.H. Rieckman, and C. J. Canfield, *Science*, 190 (1975) 792.

[20] K.H. Rieckman, G.M. Trenholme, R.L. Williams, R.E. Carson, H. Frischer, and R.Z. Desjardins, *Bull. WHO*, 51 (1974) 375.

[21] D.F. Clyde, V.C. McCarthy, R.M. Miller, and R.B. Hornick, *Antimicrob. Agents. Chemother.*, 9 (1976) 384.

[22] T. Nakagawa, T. Higuchi, J.L. Haslam, R.D. Shaffer, and W.D. Mendenhall, *J. Pharm. Sci.*, 68 (1979) 718.

[23] S.M. Mansor, V. Navaratnam, M. Mohamad, S. Hussein, A. Kumar, A. Jamaludin, W.H. Wernsdorfer, Single dose kinetic study of the triple combination mefloquine/sulphadoxine/pyrimethamine (Fansimef®) in healthy male volunteers. Br. J. Clin. Pharmacol., 27(1989)381-6.

[24] D. Dadgar, J. Climax, R. Lambe, A Darragh. Gas chromatographic determination of mefloquine in human and dog plasma using electron-capture detection. J. Chromatogr., 337(1985)47.

[25] D.E. Schwartz, in A. Frigerio and M. McCamish (Editors), Recent Developments in Chromatography and Electrophoresis, Vol. 10, Elsevier, Amsterdam, 1980, p. 69.

[26] D.E. Schwartz and U.B. Ranalder, *Biomed. Mass Spectrom.*, 8 (1981) 589.

[27] J.M. Grindel, P.F. Tilton, and R.D. Shaffer. Quantitation of the antimalarial agent, mefloquine, in blood, plasma, and urine using high-pressure liquid chromatography. *J. Pharm. Sci.*, 66:6 (1977) 834.

[28] I.M. Kapetanovic, J.D. DiGiovanni, J. Bartosevich, V. Melendez, J. von Bredow and M. Heiffer. Analysis of the antimalarial, mefloquine, in blood and plasma using high-performance liquid chromatography. *J. Chromatogr.* 277 (1983) 209.

[29] G. Franssen, B. Rouveix, J. Lebras, J. Bauchet, F. Verdier, C. Michon, F. Bricaire. Divided-dose kinetics of mefloquine in man. Br. J. Clin. Pharmacol., 28(1989)179-184.

[30] J. Karbwang, P. Molunto, K.N. Bangchang, D. Bunnag. Determination of mefloquine in biological fluids using high performance liquid chromatography. S.E. Asian J. Trop. Med. Pub. Hlth., 20:1(1989)55-60.

[31] M.C. Coleman, L. Fleckenstein, L.A. Shipley, M.H. Heiffer. Disposition of the antimalarial, mefloquine, in the isolated perfused rat liver. Biochem. Pharmacol., 37:2(1988)235-239.

[32] J.H. Riviere, D.J. Back, A.M. Breckenridge, R.E. Howells. The pharmacokinetics of mefloquine in man: lack of effect of mefloquine on antipyrine metabolism. Br. J. Clin. Pharmacol., 20(1985)469-474.

[33] Y. Bergqvist, U. Hellgren, F.C. Churchill. High-performance liquid chromatographic assay for the simultaneous monitoring of Mefloquine and its acid metabolite in biological samples using protein precipitation and ion-pair extraction. J. Chromatogr., 432(1988)253-263.

[34] R. Jauch, E. Griesser, G. Oesterhelt. Metabolismus von Ro 21-5998 (Mefloquin) bei der Ratte. Arzneim. Forsch./Drug Res., 30(1980)60-67.

[35] E.A. Nodiff, S. Chaterjee and H.A. Musallam, *Prog. Med. Chem.*, 28 (1991)1.

[36] J.M. Karle and R. Olmeda, "Rapid and Sensitive Quantitative Analysis of the New Antimalarial N^4-[2,6-Dimethoxy-4-Methyl-5-[(3-Trifluoromethyl)

phenoxy]-8-Quinolinyl]-1,4-Pentanediamine in Plasma by Liquid Chromatography and Electrochemical Detection" *J. Chromatogr.*, 424(1988) 347.

[37] E.T. Lin and W.L. Gee, "Quantitation of Primaquine (FreeBase) and its Carboxylated Metabolite in Human Plasma by High Performance Liquid Chromatography and Ultraviolet Detection," Study Report 23, Contract No. DAMD17-92-C-2028, USAMRDC, 1996.

[38] E.T. Lin, L.Z. Benet, R.A. Upton, and W.L. Gee, "Quantitation of WR 238,605 as FreeBase in Plasma and Blood by HPLC and Fluorescence Detection," Study Report 13B, Contract No. DAMD17-85-C-6150, USAMRDC, 1989.

[39] M.T. Marino, J.O. Peggins and T.G. Brewer, "High-performance liquid chromatographic method for the determination of a candidate 8-aminoquinoline antimalarial drug (WR 242511) using oxidative electrochemical detection," *J. Chromatogr. Biomedical Applications*, 616(1993)338.

[40] B.A. Bidlingmeyer, J.K. Del Rios, and J. Korpi, "Separation of Organic Amine Compounds on Silica Gel with Reversed-Phase Eluents," *Anal. Chem.* 54(1982)442.

APPENDIX A

Draft Study Report No. 29B

Validation of a Liquid Chromatographic/Mass Spectrometric/Mass Spectrometric (LC/MS/MS) Method for the Determination of Chloroquine (and its Metabolites) in Human Blood Samples
(with a Short Validation for Human Plasma Samples)

III. Methods
A. Materials

Test Compounds:	Chloroquine Monodesethylchloroquine Didesethylchloroquine
Drug Standards:	-Chloroquine (WR 1544), bottle number AU29291, was obtained from WRAIR. -Monodesethylchloroquine (WR 029623), bottle number BL11088, was obtained from WRAIR. -Didesethylchloroquine (WR 112472), bottle number BH73127, was obtained from WRAIR.
Internal Standard:	Neostigmine bromide, Lot number KT05130JT, was purchased from Aldrich Chemical Co, Milwaukee, WI.
Matrix:	Human Blood
Biological Matrix:	Control and standard curve human blood was obtained Irwin Memorial Blood Bank, San Francisco, CA. This matrix was used for daily preparation of standard and quality control samples. Prior to use, blood was stored at -20°C. This material was found to be free of endogenous substances that would interfere with the quantitation of the drug or internal standard.
Sample Storage:	Temperature: Approximately -70°C

LC/MS/MS System

Detector:	API III PE-Sciex (Perkin-Elmer, Norwalk, CT)
Pump:	Shimadzu LC-10AD Pump (Shimadzu Scientific Instruments, Inc., Columbus, MD) or equivalent
Injector:	Waters Intelligent Sample Processor 717 Plus (Waters Associates, Milford, MA) or equivalent
Data Acquisition:	Macintosh Quadra 800 (Apple, Cupertino, CA) Mac Spec 3.3 Software (Perkin-Elmer, Norwalk, CT) RAD 2.4 Software (Perkin-Elmer, Norwalk, CT)
Data Reduction:	Internal standard method using peak area ratio (PAR). Weighted (1/y) linear regression of concentration (x-axis) vs. PAR (y-axis)

LC/MS/MS Conditions

Column:	Hypersil Silica, 3 µm particle size, 4.6 X 50 mm (Keystone Scientific, Inc., Bellefonte, PA) or equivalent
Column Temperature:	Room Temperature
Mobile Phase:	90% acetonitrile, 0.1% trifluoroacetic acid (TFA), 5 mM ammonium acetate. The mobile phase was prepared by mixing 3600 ml of CH_3CN with 400 ml of water, adding 4 ml of TFA and adding 10 ml of 2M CH_3COONH_4 to yield about 4 liters. The resulting solution was filtered through a 5 micron filter and degassed under vacuum prior to use.
Flow Rate:	1.2 ml/min.
Sample Inlet Mode:	Heated Nebulizer
Ionization Mode:	APCI/Positive Ionization
Discharge Current:	+ 3 µA
Curtain Gas Flow Rate:	1.2 L/min (N_2 = 99.999%)
Nebulizer Pressure:	80 psi (Ultra Zero Air)
Auxiliary Flow Rate:	2.0 L/min (Ultra Zero Air)
CAD Gas:	250 x 10^{12} molecules/cm^2 (9.99% N_2/Ar)
Interface Heater Temperature:	55°C
Heated Nebulizer Temperature Controller:	480-500°C
Laboratory Temperature:	60-80°F

Note: If necessary, the LC/MS/MS conditions can be slightly modified to optimize the system.

Mass Scanning Mode: MRM (Multiple Reaction Monitoring)

Chloroquine:	321 - 247 *m/z*
Monodesethylchloroquine:	292 - 114 *m/z*
Didesethylchloroquine:	265 - 179 *m/z*
Neostigmine Br (I.S.):	209 - 72 *m/z*

Assay Parameters

Volume of Blood Required for Assay:	100 µl
Assay Ranges:	Chloroquine and Metabolites: 20.0 to 2000 ng/ml
Minimum Reportable Concentrations:	20.0 ng/ml for Chloroquine and Metabolites

Chemicals and Supplies

Chemical/Solvents	Grade	Supplier
Acetonitrile	HPLC	Fisher Scientific
Ammonium acetate	HPLC	Fisher Scientific
Trifluoroacetic Acid	Reagent	Sigma Chemical
Acetic Acid, Glacial	Reagent	Fisher Scientific
Water	Type I Reagent Grade	Nanopure, Barnstead
Methanol	Optima	Fisher Scientific

B. Analytical Method

Human blood samples (100 µl) were analyzed for chloroquine, monodesethylchloroquine, and didesethylchloroquine with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a silica column (4.6 x 50 mm, 3 µm particle size), 90% CH_3CN, 0.1% trifluoroacetic acid (TFA) and 5 mM ammonium acetate mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (atmospheric pressure chemical ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of 50 µl of water, sonication of the mixture for 5 minutes, addition of 400 µl of acetonitrile containing neostigmine internal standard (IS), mixing of the mixture for 1 minute, centrifugation for 10 minutes and transfer of the supernatant prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free human blood samples with known amounts of chloroquine monodesethylchloroquine, didesethylchloroquine, and IS. Standard curve, QC and assay samples were prepared as described, then ~2 µl aliquots were injected into the LC/MS/MS system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios of chloroquine (daughter ion at 247 *m/z* from parent ion at 321 *m/z*) and monodesethylchloroquine (daughter ion at 114 *m/z* from parent ion at 292 *m/z*), didesethylchloroquine (daughter ion at 179 *m/z* from parent ion at 265 *m/z*), (daughter ion at 251 *m/z* from parent ion at 325 *m/z*) to IS (daughter ion at 72 *m/z* from parent ion at 209 *m/z*) were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations of chloroquine, monodesethyl-chloroquine, and didesethylchloroquine to IS peak area ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the three equations for the best straight lines ($y = mx + b$, where y = peak area ratio and x = chloroquine, monodesethyl-chloroquine, or didesethylchloroquine concentrations), and drug and metabolite concentrations in assay samples were calculated by these equations from the chloroquine, monodesethylchloroquine, and didesethylchloroquine to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank human blood with chloroquine, monodesethyl-chloroquine, and didesethylchloroquine.

Retention times (approximate, in minutes) were internal standard 1:22, chloroquine 1:54, monodesethylchloroquine 1:40, didesethylchloroquine 1:40. The time between injections was 2-3 minutes.

Standard and Control Solutions

STOCK SOLUTIONS: These solutions were stored in a 4°C refrigerator. The chloroquine (CHL), monodesethylchloroquine (MDC), and didesethyl-chloroquine (DDC) were protected from light.

Solution Type	Weight of Standard (mg)	Adjustment Factor*	QS Volume (ml)	Solvent	Conc. (mg/ml)
CHL Standard Curve	8.18	0.6200	5.072	50% CH_3OH	1.00
MDC Standard Curve	8.12	0.6185	5.022	50% CH_3OH	1.00
DDC Standard Curve	6.62	0.7656	5.068	50% CH_3OH	1.00
CHL Control	8.26	0.6200	5.121	50% CH_3OH	1.00
MDC Control	8.26	0.6185	5.109	50% CH_3OH	1.00
DDC Control	6.56	0.7656	5.022	50% CH_3OH	1.00
Neostigmine Internal Standard	10.38	0.982	101.93	50% CH_3OH	0.100

* = Molecular weights of chloroquine free base/chloroquine diphosphate, monodesethyl-chloroquine free base/monodesethylchloroquine dioxalate, didesethylchloroquine free base/didesethylchloroquine hydrobromide or neostigmine free base/neostigmine bromide.

WORKING SOLUTIONS. These solutions were stored in a 4°C refrigerator and protected against light (neostigmine solution was not protected). CHL, MDC, DDC, stock solutions were combined into a single solution (high working solution-HWS), diluted with diluting solution (40% methanol, 40 mM ammonium acetate, 0.1% acetic acid) and QS to 25.0. The low working solutions (LWS) were also generated with the diluting solution.

Solution Type	Volume Diluted (ml)	Conc. Diluted (mg/ml)	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve Substock [CHL]	1.00	1.00	10.0	diluting solution	100
Standard Curve Substock [MDC]	1.00	1.00	10.0	diluting solution	100
Standard Curve Substock [DDC]	1.00	1.00	10.0	diluting solution	100
Standard Curve HWS [CHL , MDC, DDC]	0.500	0.100	10.0	diluting solution	5.00
Standard Curve LWS [CHL , MDC, DDC]	1.00	0.005	10.0	diluting solution	0.500
Control Substock [CHL]	1.00	1.00	10.0	diluting solution	100
Control Substock [MDC]	1.00	1.00	10.0	diluting solution	100
Control Substock [DDC]	1.00	1.00	10.0	diluting solution	100
Control HWS [CHL , MDC, DDC]	0.500	0.100	10.0	diluting solution	5.00
Control LWS [CHL , MDC, DDC]	1.00	0.005	10.0	diluting solution	0.500
Neostigmine Working Internal Standard	0.5	100	500	CH_3CN	0.100

Calibration Standards and Quality Control Samples

The scheme for generating calibration standard and quality control (QC) samples for chloroquine, monodesethylchloroquine, and didesethylchloroquine is provided in the following tables.

Calibration Standards: Calibration standards were generated by spiking 0.100 ml blank human blood specimens with chloroquine, monodesethylchloroquine, and didesethylchloroquine standard curve solutions. This procedure is equivalent to addition of the masses of chloroquine, monodesethylchloroquine, and didesethylchloroquine shown below. Since 0.100 ml blood samples are assayed, these amounts correspond to the nominal concentrations shown below. Vortex for 10 seconds.

Generation of Chloroquine, Monodesethylchloroquine, and Didesethylchloroquine Standard Curve Calibrators

Sample	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	CHL, MDC, and DDC Mass Spiked (ng)	CHL, MDC, and DDC Standard Curve Sample Nominal Concentration (ng/ml)
00*	0	0	0	0
0**	0	0	0	0
1	4.00	0.500	2.00	20.0
2	8.00	0.500	4.00	40.0
3	16.0	0.500	8.00	80.0
4	30.0	0.500	15.0	150
5	6.00	5.00	30.0	300
6	10.0	5.00	50.0	500
7	20.0	5.00	100	1000
8	40.0	5.00	200	2000

Quality Control Samples: Quality control samples were generated by spiking 100 µl blank human blood specimens as follows.

Generation of Chloroquine, Monodesethylchloroquine, and Didesethylchloroquine Precision Quality Control Samples

	Volume Spiked (µl)	Spiking Solution Conc. (µg/ml)	Blood Volume (µl)	CHL, MDC, and DDC Quality Control Sample Nominal Conc. (ng/ml)
Low	8.00	LWS	100	40.0
Medium	10.0	HWS	100	500
High	30.0	HWS	100	1500

SAMPLE PREPARATION PROCEDURE

1. Pipet 100 µl of human blood into a 13 x 100 silanized tube.
2. Spike standard curve samples with chloroquine, monodesethyl-chloroquine, and didesethylchloroquine as described above and let stand at room temperature for 30 minutes.
3. Add 50 µl of nanopure water.
4. Sonicate mixture for 5 minutes.
5. Add 400 µl of internal standard working solution (100 ng/ml neostigmine bromide in acetonitrile) and vortex 1 minute.
6. Centrifuge for 10 minutes at 3000 rpm.
7. Transfer supernatant to WISP vial.
8. Inject 2 µl onto the column.

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* 00 = Sample with no drug and no internal standard.
** 0 = Sample with no drug but with internal standard.

Study Report No. 31

Validation of a Liquid Chromatographic/Mass Spectrometric/Mass Spectrometric (LC/MS/MS) Method for the Determination of Chloroquine (and its metabolites), Quinine, Doxycycline, Halofantrine (and its metabolite), Mefloquine, and WR 238,605 in Dog Plasma Samples, Part I: WR 238,605 and Mefloquine

III. Methods

A. Materials

Test Compounds: WR 238,605
Mefloquine

Drug Standards: WR 238,605, Bottle number BM12562, was obtained from WRAIR.
Mefloquine, Bottle number BK 115921, was obtained from WRAIR.

Internal Standard: Verapamil, Lot number 68F-0758, was purchased from Sigma Chemical Co, St. Louis, MO.

Matrix: Dog Plasma

Biological Matrix: Control and standard curve dog plasma was obtained from Pel-Freez Biologicals, Rogers, AK. This matrix was used for daily preparation of standard and quality control samples. Prior to use, plasma was stored at -20°C. This material was found to be free of endogenous substances that would interfere with the quantitation of the drug or internal standard.

Sample Storage: Temperature: Approximately -70°C

LC/MS/MS System

Detector: API III PE-Sciex (Perkin-Elmer, Norwalk, CT)

Pump: Shimadzu LC-10AD Pump (Shimadzu Scientific Instruments, Inc., Columbus, MD) or equivalent

Injector: Waters Intelligent Sample Processor 717 Plus (Waters Associates, Milford, MA) or equivalent

Data Acquisition: Macintosh Quadra 800 (Apple, Cupertino, CA)
Mac Spec 3.3 Software (Perkin-Elmer, Norwalk, CT)
RAD 2.4 Software (Perkin-Elmer, Norwalk, CT)

Data Reduction: Internal standard method using peak area ratio (PAR). Weighted (1/y) linear regression of concentration (x-axis) vs. PAR (y-axis)

LC/MS/MS Conditions

Column:	Hypersil Silica, 3 µm particle size, 4.6 X 50 mm (Keystone Scientific, Inc., Bellefonte, PA) or equivalent
Column Temperature:	Room Temperature
Mobile Phase:	90% CH_3CN and 0.06% trifluoroacetic acid. The mobile phase was prepared by mixing 3600 ml of acetonitrile with 400 ml of water, adding 2.4 ml of trifluoroacetic acid to yield approximately 4 liters. The resulting solution was filtered through a 5 micron filter and degassed under vacuum prior to use.
Flow Rate:	1.3 ml/min.
Sample Inlet Mode:	Heated Nebulizer
Ionization Mode:	APCI/Positive Ionization
Discharge Current:	+ 3 µA
Curtain Gas Flow Rate:	1.2 L/min. (N_2 = 99.999%)
Nebulizer Pressure:	80 psi (Ultra Zero Air)
Auxiliary Flow Rate:	2.0 L/min. (Ultra Zero Air)
CAD Gas:	250 x 10^{12} molecules/cm^2 (9.99% N_2/Ar)
Interface Heater Temperature:	55°C
Heated Nebulizer Temperature Controller:	450°C

Note: If necessary, the LC/MS/MS conditions can be slightly modified to optimize the system.

Mass Scanning Mode: MRM (Multiple Reaction Monitoring)

Mefloquine:	379 - 359 *m/z*
WR 238,605:	464 - 86 *m/z*
Internal Standard:	455 - 165 *m/z*

Assay Parameters

Volume of Plasma Required for Assay:	200 µl
Assay Ranges:	WR 238,605: 4.00 to 800 ng/ml Mefloquine: 5.00 to 1000 ng/ml
Minimum Reportable Concentrations:	WR 238,605: 4.00 ng/ml Mefloquine: 5.00 ng/ml

Chemicals and Supplies

Chemical/Solvents	Grade	Supplier
Acetonitrile	HPLC	Fisher Scientific
Trifluoroacetic Acid	Reagent	Sigma Chemical
Water	Type I Reagent Grade	Nanopure, Barnstead
Methanol	Optima	Fisher Scientific
Ammonium Acetate	HPLC	Fisher Scientific
Acetic Acid, Glacial	Reagent	Fisher Scientific

B. Analytical Method

Dog plasma samples (200 µl) were analyzed for WR 238,605 and mefloquine with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a silica column (4.6 x 50 mm, 3 µm particle size) and 90% CH_3CN, 0.06% trifluoroacetic acid (TFA) mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (atmospheric pressure chemical ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of 100 µl of methanol, addition of verapamil internal standard (IS), and transfer of the supernatant prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free dog plasma samples with known amounts of WR 238,605, mefloquine, and IS. Standard curve, QC and assay samples were prepared as described, then ~5 µl aliquots were injected into the LC/MS/MS system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios for WR 238,605 (daughter ion at 86 *m/z* from parent ion at 464 *m/z*) and mefloquine (daughter ion at 359 *m/z* from parent ion at 379 *m/z*) to IS (daughter ion at 165 *m/z* from parent ion at 455 *m/z*) were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations and WR 238,605 and mefloquine to IS peak area ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the two equations for the best straight lines ($y = mx + b$, where y = peak area ratio and x = WR 238,605 or mefloquine concentrations), and drug concentrations in assay samples were calculated by these equations from the WR 238,605 and mefloquine to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank dog plasma with WR 238,605 and mefloquine.

Standard Curve and Control Solutions

STOCK SOLUTIONS: These solutions were stored in a 4°C refrigerator. The WR 238,605 and mefloquine were protected from light.

Solution Type	Weight of Standard (mg)	Adjustment Factor*	QS Volume (ml)	Solvent	Conc. (mg/ml)
WR 238,605 Standard Curve	6.27	0.7971	50.0	CH_3OH	0.100
Mefloquine Standard Curve	11.05	0.912	10.078	50% CH_3OH	1.00
WR 238,605 Control	6.40	0.7971	51.01	CH_3OH	0.100
Mefloquine Control	11.0	0.912	10.032	50% CH_3OH	1.00
Verapamil (IS)	10.04	1.00	10.04	50% CH_3OH	1.00

* Molecular weights of WR 238605 free base/WR 238605 succinate and mefloquine free base/mefloquine HCl.

WORKING SOLUTIONS. These solutions were stored in a 4°C refrigerator and protected against light. WR 238,605 and Mefloquine stock solutions were combined into a single solution (high working solution, HWS), diluted with diluting solution (40% methanol, 40 mM ammonium acetate, 0.1% acetic acid) and QS to 10.0 ml to make 8.00 µg/ml WR 238,605 and 10.0 µg/ml Mefloquine concentrations. The low working solutions (LWS) were also diluted with diluting solution and QS to 10.0 ml to make 0.800 µg/ml WR 238,605 and 1.00 µg/ml Mefloquine concentrations.

Solution Type	Volume Diluted (ml)	Conc. Diluted (µg/ml)	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve HWS					
[WR 238,605]	0.800	100	10.0	diluting solution	8.00
[Mefloquine]	0.100	1000			10.0
Standard Curve LWS					
[WR 238,605]	1.00	8.00	10.0	diluting solution	0.800
[Mefloquine]		10.0			1.00
Control HWS					
[WR 238,605]	0.800	100	10.0	diluting solution	8.00
[Mefloquine]	0.100	1000			10.0
Control LWS					
[WR 238,605]	1.00	8.00	10.0	diluting solution	0.800
[Mefloquine]		10.0			1.00
Verapamil Substock (IS)	0.005	1000	100	CH_3CN	0.050
Verapamil Working (IS)	10.0	0.050	100	CH_3CN	0.005

NOTE: The working solutions may also include Chloroquine, Monodesethylchloroquine, Didesethylchloroquine, Quinine, and/or Doxycycline, if these other chemicals need to be assayed in the same study. The Internal Standard solution may also include Neostigmine Br and/or Minocycline, if Chloroquine, Monodesethylchloroquine, Didesethylchloroquine, Quinine and/or Doxycycline need to be assayed in the same study. See the appropriate validation report for volume and concentration information.

Calibration Standards and Quality Control Samples

The scheme for generating calibration standard and quality control (QC) samples for WR 238,605 and Mefloquine is provided in the following tables.

Calibration Standards: Calibration standards were generated by spiking 0.200 ml blank dog plasma specimens with WR 238,605 and Mefloquine standard curve solutions. This procedure is equivalent to addition of the masses of WR 238,605 and Mefloquine shown below. Since 0.200 ml plasma samples are assayed, these amounts correspond to the nominal concentrations shown below. Vortex for 10 seconds.

Generation of WR 238,605 and Mefloquine Standard Curve Calibrators

Sample	Volume Spiked (µl)	Spiking Solution Conc. (µg/ml)	WR 238,605 Mass Spiked (ng)	Mefloquine Mass Spiked (ng)	WR 238,605 Standard Curve Sample Nominal Conc. (ng/ml)	Mefloquine Standard Curve Sample Nominal Conc. (ng/ml)
00*	0.00	0	0	0	0	0
0**	0.00	0	0	0	0	0
1	1.00	LWS	0.800	1.00	4.00	5.00
2	2.00	LWS	1.60	2.00	8.00	10.0
3	4.00	LWS	3.20	4.00	16.0	20.0
4	8.00	LWS	6.40	8.00	32.0	40.0
5	15.0	LWS	12.0	15.0	60.0	75.0
6	3.00	HWS	24.0	30.0	120	150
7	5.00	HWS	40.0	50.0	200	250
8	10.0	HWS	80.0	100	400	500
9	20.0	HWS	160	200	800	1000

Quality Control Samples: Quality control samples were generated by spiking 200 µl blank dog plasma specimens as follows.

Generation of WR 238,605 and Mefloquine Precision Quality Control Samples

	Volume Spiked (µl)	Spiking Solution Conc. (µg/ml)	Plasma Volume (µl)	WR 238,605 Quality Control Sample Nominal Conc. (ng/ml)	Mefloquine Quality Control Sample Nominal Conc. (ng/ml)
X-Low	2.00	LWS	200	8.00	10.0
Low	10.0	LWS	200	40.0	50.0
Med.	4.00	HWS	200	160	200
High	15.0	HWS	200	600	750

SAMPLE PREPARATION PROCEDURE

* Sample with no drug and no internal standard.

** Sample with no drug, but with internal standard.

1. Pipette 200 µl of blank dog plasma into a 13 x 100 silanized tube.

2. Spike standard curve samples with WR 238,605 and Mefloquine as described above.

3. Add 100 µl of methanol. Vortex for 1 minute.

4. Add 500 µl of internal standard working solution (5.00 ng/ml verapamil) and vortex 1 minute.

5. Centrifuge for 5 minutes at 3000 rpm.

6. Transfer ~300 µl of supernatant to WISP vial and inject 5 µl onto the column.

Study Report No. 31

Validation of a Liquid Chromatographic/Mass Spectrometric/Mass Spectrometric (LC/MS/MS) Method for the Determination of Chloroquine (and its Metabolites), Quinine, Doxycycline, Halofantrine (and its Metabolite), Mefloquine, and WR 238,605 in Dog Plasma Samples, Part II: Chloroquine and Quinine

III. Methods

A. Materials

Test Compounds: Chloroquine
Monodesethylchloroquine
Didesethylchloroquine
Quinine

Drug Standards: Chloroquine (WR 1544), bottle number AU29291, was obtained from WRAIR.
Monodesethyl-chloroquine (WR 029623), bottle number BL11088, was obtained from WRAIR.
Didesethyl-chloroquine (WR 112472), bottle number BH73127, was obtained from WRAIR.
Quinine, bottle number 1826KE, was obtained from Aldrich Chemical Co, Milwaukee, WI.

Internal Standard: Neostigmine bromide, Lot number KT05130JT, was purchased from Aldrich Chemical Co, Milwaukee, WI.

Matrix: Dog Plasma (*Canis familiaris*)

Biological Matrix: Control and standard curve dog plasma was obtained from Pel-Freez Biologicals, Rogers, AK. This matrix was used for daily preparation of standard and quality control samples. Prior to use, plasma was stored at -20°C. This material was found to be free of endogenous substances that would interfere with the quantitation of the drug or internal standard.

Sample Storage: Temperature: Approximately -70°C

LC/MS/MS System

Detector: API III PE-Sciex (Perkin-Elmer, Norwalk, CT)

Pump: Shimadzu LC-10AD Pump (Shimadzu Scientific Instruments, Inc., Columbus, MD) or equivalent

Injector: Waters Intelligent Sample Processor 717 Plus (Waters Associates, Milford, MA) or equivalent

Data Acquisition: Macintosh Quadra 800 (Apple, Cupertino, CA)
Mac Spec 3.3 Software (Perkin-Elmer, Norwalk, CT)
RAD 2.4 Software (Perkin-Elmer, Norwalk, CT)

Data Reduction: Internal standard method using peak area ratio (PAR). Weighted (1/y) linear regression of concentration (x-axis) vs. PAR (y-axis)

LC/MS/MS Conditions

Column:	Hypersil Silica, 3 µm particle size, 4.6 X 50 mm (Keystone Scientific, Inc., Bellefonte, PA) or equivalent
Column Temperature:	Room Temperature
Mobile Phase:	90% acetonitrile, 0.1% trifluoroacetic acid (TFA), 5 mM ammonium acetate. The mobile phase was prepared by mixing 3600 ml of CH_3CN with 400 ml of water, adding 4 ml of TFA and adding 10 ml of 2M CH_3COONH_4 to yield approximately 4 liters. The resulting solution was filtered through a 5 micron filter and degassed under vacuum prior to use.
Flow Rate:	1.3 ml/min.
Sample Inlet Mode:	Heated Nebulizer
Ionization Mode:	APCI/Positive Ionization
Discharge Current:	+ 3 µA
Curtain Gas Flow Rate:	1.2 L/min (N_2 = 99.999%)
Nebulizer Pressure:	80 psi (Ultra Zero Air)
Auxiliary Flow Rate:	2.0 L/min (Ultra Zero Air)
CAD Gas:	250 x 10^{12} molecules/cm^2 (9.99% N_2/Ar)
Interface Heater Temperature:	55°C
Heated Nebulizer Temperature Controller:	500°C
Laboratory Temperature:	60-80°F

Note: If necessary, the LC/MS/MS conditions can be slightly modified to optimize the system.

Mass Scanning Mode: MRM (Multiple Reaction Monitoring)

Chloroquine:	321 - 247 *m/z*
Monodesethylchloroquine:	292 - 114 *m/z*
Didesethylchloroquine:	265 - 179 *m/z*
Quinine:	325 - 251 *m/z*
Neostigmine Br (I.S.):	209 - 72 *m/z*

Assay Parameters

Volume of Plasma Required for Assay:	200 µl
Assay Ranges:	Chloroquine and Metabolites: 4.00 to 800 ng/ml Quinine: 10.0 to 2000 ng/ml
Minimum Reportable Concentrations:	4.00 ng/ml for Chloroquine and Metabolites 10.0 ng/ml for Quinine

Chemicals and Supplies

Chemical/Solvents	**Grade**	**Supplier**
Acetonitrile	HPLC	Fisher Scientific
Ammonium acetate	HPLC	Fisher Scientific
Trifluoroacetic Acid	Reagent	Sigma Chemical
Acetic Acid, Glacial	Reagent	Fisher Scientific
Water	Type I Reagent Grade	Nanopure, Barnstead
Methanol	Optima	Fisher Scientific

B. Analytical Method

Dog plasma samples (200 µl) were analyzed for chloroquine, monodesethyl-chloroquine, didesethylchloroquine, and quinine with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a silica column (4.6 x 50 mm, 3 µm particle size), 90% CH_3CN, 0.1% trifluoroacetic acid (TFA) and 5 mM ammonium acetate mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (atmospheric pressure chemical ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of 100 µl of methanol and of neostigmine internal standard (IS), mixing of the mixture, centrifugation and transfer of the supernatant prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free dog plasma samples with known amounts of chloroquine, monodesethyl-chloroquine, didesethylchloroquine, quinine, and IS. Standard curve, QC and assay samples were prepared as described, then ~8 µl aliquots were injected into the LC/MS/MS system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios of chloroquine (daughter ion at 247 *m/z* from parent ion at 321 *m/z*) and monodesethylchloroquine (daughter ion at 114 *m/z* from parent ion at 292 *m/z*), didesethylchloroquine (daughter ion at 179 *m/z* from parent ion at 265 *m/z*), and quinine (daughter ion at 251 *m/z* from parent ion at 325 *m/z*) to IS (daughter ion at 209 *m/z* from parent ion at 72 *m/z*) were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations of chloroquine, monodesethyl-chloroquine, didesethylchloroquine, and quinine to IS peak area ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the two equations for the best straight lines ($y = mx + b$, where y = peak area ratio and x = chloroquine, monodesethylchloroquine, didesethyl-chloroquine, or quinine concentrations), and drug and metabolite concentrations in assay

samples were calculated by these equations from the chloroquine, monodesethylchloroquine, didesethylchloroquine, and quinine to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank dog plasma with chloroquine, monodesethyl-chloroquine, didesethylchloroquine, and quinine.

Retention times (in minutes) were internal standard 1:12-14, chloroquine 1:39-40, monodesethylchloroquine 1:24-27, didesethylchloroquine1:25-28, and quinine1:03-09. The time between injections was 2-3 minutes.

Standard and Control Solutions

STOCK SOLUTIONS: These solutions were stored in a 4°C refrigerator. The chloroquine (CHL), monodesethylchloroquine (MDC), didesethyl-chloroquine (DDC), and quinine were protected from light.

Solution Type	Weight of Standard (mg)	Adjustment Factor*	QS Volume (ml)	Solvent	Conc. (mg/ml)
CHL Standard Curve	1.71	0.6200	10.0	50% CH_3OH	0.106
MDC Standard Curve	1.77	0.6185	10.0	50% CH_3OH	0.109
DDC Standard Curve	1.36	0.7656	10.0	50% CH_3OH	0.104
Quinine Standard Curve	12.04	0.8483	10.214	50% CH_3OH	1.00
CHL Control	1.86	0.6200	10.0	50% CH_3OH	0.115
MDC Control	1.66	0.6185	10.0	50% CH_3OH	0.103
DDC Control	1.33	0.7656	10.0	50% CH_3OH	0.102
Quinine Control	11.96	0.8483	10.145	50% CH_3OH	1.00
Neostigmine Internal Standard	5.21	0.982	51.16	50% CH_3OH	0.100

* = Molecular weights of chloroquine free base/chloroquine diphosphate, monodesethyl-chloroquine free base/monodesethylchloroquine dioxalate, didesethylchloroquine free base/didesethylchloroquine hydrobromide or neostigmine free base/neostigmine bromide.

WORKING SOLUTIONS. These solutions were stored in a 4°C refrigerator and protected against light (neostigmine solution was not protected). CHL, MDC, DDC, and quinine stock solutions were combined into a single solution (high working solution-HWS), diluted with diluting solution (40% methanol, 40 mM ammonium acetate, 0.1% acetic acid) and QS to 10.0 ml to make 8.00 µg/ml CHL, MDC, and DDC and 20.0 µg/ml Quinine concentrations. The low working solutions (LWS) were generated with the diluting solution.

Solution Type	Volume Diluted (ml)	Conc. Diluted (µg/ml)	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve HWS [CHL]	0.754	106	10.0	diluting solution	8.00
Standard Curve HWS [MDC]	0.734	109	10.0	diluting solution	8.00
Standard Curve HWS [DDC]	0.770	104	10.0	diluting solution	8.00
Standard Curve HWS [Quinine]	0.200	1000	10.0	diluting solution	20.0
Standard Curve LWS [CHL , MDC, DDC] [Quinine]	1.00	HWS	10.0	diluting solution	0.800 2.00
Control HWS [CHL]	0.696	115	10.0	diluting solution	8.00
Control HWS [MDC]	0.776	103	10.0	diluting solution	8.00
Control HWS [DDC]	0.784	103	10.0	diluting solution	8.00
Control HWS [Quinine]	0.200	1000	10.0	diluting solution	20.0
Control LWS [CHL , MDC, DDC] [Quinine]	1.00	HWS	10.0	diluting solution	0.800 2.00
Neostigmine Working Internal Standard	0.025	100	100	CH_3CN	0.025

Calibration Standards and Quality Control Samples

The scheme for generating calibration standard and quality control (QC) samples for chloroquine, monodesethylchloroquine, didesethylchloroquine, and quinine is provided in the following tables.

Calibration Standards: Calibration standards were generated by spiking 0.200 ml blank dog plasma specimens with chloroquine, monodesethyl-chloroquine, didesethylchloroquine, and quinine standard curve solutions. This procedure is equivalent to addition of the masses of chloroquine, monodesethylchloroquine, didesethylchloroquine, and quinine shown below. Since 0.200 ml plasma samples are assayed, these amounts correspond to the nominal concentrations shown below. Vortex for 10 seconds.

Generation of Chloroquine, Monodesethylchloroquine, Didesethylchloroquine, and Quinine Standard Curve Calibrators

Sample	Volume Spiked (µl)	Spiking Solution Conc. (µg/ml)	CHL, MDC, and DDC Mass Spiked (ng)	Quinine Mass Spiked (ng)	CHL, MDC, and DDC Standard Curve Sample Nominal Conc. (ng/ml)	Quinine Standard Curve Sample Nominal Conc. (ng/ml)
00*	0.00	0	0	0	0	0
0**	0.00	0	0	0	0	0
1	1.00	LWS	0.800	2.00	4.00	10.0
2	2.00	LWS	1.60	4.00	8.00	20.0
3	4.00	LWS	3.20	8.00	16.0	40.0
4	8.00	LWS	6.40	16.0	32.0	80.0
5	15.0	LWS	12.0	30.0	60.0	150
6	3.00	HWS	24.0	60.0	120	300
7	5.00	HWS	40.0	100	200	500
8	10.0	HWS	80.0	200	400	1000
9	20.0	HWS	160	400	800	2000

Quality Control Samples: Quality control samples were generated by spiking 200 µl blank dog plasma specimens as follows.

Generation of Chloroquine and Monodesethylchloroquine, Didesethylchloroquine, and Quinine Precision Quality Control Samples

	Volume Spiked (µl)	Spiking Solution Conc. (µg/ml)	Plasma Volume (µl)	CHL, MDC, and DDC Quality Control Sample Nominal Conc. (ng/ml)	Quinine Quality Control Sample Nominal Conc. (ng/ml)
X-Low	2.00	LWS	200	8.00	20.0
Low	10.0	LWS	200	40.0	100
Med.	4.00	HWS	200	160	400
High	15.0	HWS	200	600	1500

SAMPLE PREPARATION PROCEDURE

1. Pipet 200 µl of blank dog plasma into a 13 x 100 silanized tube.
2. Spike standard curve samples with chloroquine, monodesethyl-chloroquine, didesethylchloroquine, and quinine as described above.
3. Add 100 µl of methanol. Vortex for 1 minute.
4. Add 500 µl of internal standard working solution (25.0 ng/ml neostigmine) and vortex 1 minute.
5. Centrifuge for 5 minutes at 3000 rpm.
6. Transfer ~300 µl of supernatant to WISP vial.
7. Inject 8 µl onto the column.

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* Sample with no drug and no internal standard.
** Sample with no drug, but with internal standard.

Study Report No. 31

Validation of a Liquid Chromatographic/Mass Spectrometric/Mass Spectrometric (LC/MS/MS) Method for the Determination of Chloroquine (and its metabolites), Quinine, Doxycycline, Halofantrine (and its metabolite), Mefloquine, and WR 238,605 in Dog Plasma Samples, Part III: Doxycycline

III. Methods

A. Materials

Test Compounds:	Doxycycline
Drug Standards:	Doxycycline, Bottle number 65H-0779, was purchased from Sigma Chemical Co, St. Louis, MO.
Internal Standard:	Minocycline, Lot number 36H0473, was purchased from Sigma Chemical Co, St. Louis, MO.
Matrix:	Dog Plasma
Biological Matrix:	Control and standard curve dog plasma was obtained from Pel-Freez Biologicals, Rogers, AK. This matrix was used for daily preparation of standard and quality control samples. Prior to use, plasma was stored at -20°C. This material was found to be free of endogenous substances that would interfere with the quantitation of the drug or internal standard.
Sample Storage:	Temperature: Approximately -70°C

LC/MS/MS System

Detector:	API III PE-Sciex (Perkin-Elmer, Norwalk, CT)
Pump:	Shimadzu LC-10AD Pump (Shimadzu Scientific Instruments, Inc., Columbus, MD) or equivalent
Injector:	Waters Intelligent Sample Processor 717 Plus (Waters Associates, Milford, MA) or equivalent
Data Acquisition:	Macintosh Quadra 800 (Apple, Cupertino, CA) Mac Spec 3.3 Software (Perkin-Elmer, Norwalk, CT) RAD 2.4 Software (Perkin-Elmer, Norwalk, CT)
Data Reduction:	Internal standard method using peak area ratio (PAR). Weighted (1/y) linear regression of concentration (x-axis) vs. PAR (y-axis)

LC/MS/MS Conditions

Column:	Hypersil C8, 3 µm particle size, 4.6 X 50 mm (Keystone Scientific, Inc., Bellefonte, PA) or equivalent
Column Temperature:	Room Temperature
Mobile Phase:	35% CH_3CN and 0.1% trifluoroacetic acid, and 0.01% $(NH_4)_2HPO_4$. The mobile phase was prepared by mixing 1400 ml of acetonitrile with 2600 ml of water, adding 4 ml of trifluoroacetic acid, and 400 µl of 0.1% $(NH_4)_2HPO_4$ solution to yield approximately 4 liters. The resulting solution was filtered through a 5 micron filter and degassed under vacuum prior to use.
Flow Rate:	1.0 ml/min.
Sample Inlet Mode:	Heated Nebulizer
Ionization Mode:	APCI/Positive Ionization
Discharge Current:	+ 3 µA
Curtain Gas Flow Rate:	1.2 L/min (N_2 = 99.999%)
Nebulizer Pressure:	80 psi (Ultra Zero Air)
Auxiliary Flow Rate:	2.0 L/min (Ultra Zero Air)
CAD Gas:	250 x 10^{12} molecules/cm^2 (9.99% N_2/Ar)
Interface Heater Temperature:	55°C
Heated Nebulizer Temperature Controller:	450°C

Note: If necessary, the LC/MS/MS conditions can be slightly modified to optimize the system.

Mass Scanning Mode: MRM (Multiple Reaction Monitoring)
Doxycycline: 445 - 428 *m/z*
Minocycline (I.S.): 458 - 441 *m/z*

Assay Parameters

Volume of Plasma Required for Assay:	200 µl
Assay Ranges:	Doxycycline: 50.0 to 12800 ng/ml
Minimum Reportable Concentrations:	Doxycycline: 50.0 ng/ml

Chemicals and Supplies

Chemical/Solvents	Grade	Supplier
Acetonitrile	HPLC	Fisher Scientific
Trifluoroacetic Acid	Reagent	Sigma Chemical
Water	Type I Reagent Grade	Nanopure, Barnstead
Methanol	Optima	Fisher Scientific
Ammonium Phosphate, dibasic	Reagent	Aldrich Chemical

B. Analytical Method

Dog plasma samples (200 µl) were analyzed for doxycycline with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a C8 column (4.6 x 50 mm, 3 µm particle size) and 35% CH_3CN, 0.1% trifluoroacetic acid (TFA), and 0.01% ammonium phosphate mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (atmospheric pressure chemical ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of 100 µl of methanol, addition of minocycline internal standard (IS), and transfer of the supernatant prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free dog plasma samples with known amounts of doxycycline and IS. Standard curve, QC and assay samples were prepared as described, then injected into the LC/MS/MS system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios of doxycycline to IS were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations and doxycycline to IS peak area ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the two equations for the best straight lines ($y = mx + b$, where y = peak area ratio and x = doxycycline concentrations), and drug concentrations in assay samples were calculated by these equations from the doxycycline to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank dog plasma with doxycycline.

Standard and Control Solutions

STOCK SOLUTIONS: These solutions were stored in a 4°C refrigerator. The doxycycline were protected from light.

Solution Type	Weight of Standard (mg)	Purity factor	Conversion factor	QS Volume (ml)	Solvent	Conc. (mg/ml)
Doxycycline Standard Curve	11.9	0.924	0.939	10.325	50% CH_3OH	1.00
Doxycycline Control	11.69	0.924	0.939	10.143	50% CH_3OH	1.00
Minocycline Internal Standard	11.25	0.926	1	10.418	50% CH_3OH	1.00

WORKING SOLUTIONS. These solutions were stored in a 4°C refrigerator and protected against light.

Solution Type	Volume Diluted (ml)	Conc. Diluted (µg/ml)	QS Volume (ml)	Solvent	Conc. (µg/ml)
Doxycycline Standard Curve High Working Solution	1.00	1000	10.0	50% CH_3OH	100
Doxycycline Standard Curve Low Working Solution	1.00	100	10.0	50% CH_3OH	10.0
Doxycycline Control High Working Solution	1.00	1000	10.0	50% CH_3OH	100
Doxycycline Control Low Working Solution	1.00	100	10.0	50% CH_3OH	10.0
Minocycline Substock Internal Standard	0.500	1000	100	CH_3CN	5.00
Minocycline Working Internal Standard	10.0	5.00	100	CH_3CN	0.500

NOTE: The working solutions may also include WR 238,605, Mefloquine, Chloroquine, Monodesethyl-chloroquine, Didesethyl-chloroquine, and/or Quinine, if these other chemicals need to be assayed in the same study. The Internal Standard solution may also include Neostigmine Br and Minocycline, if Chloroquine, Monodesethyl-chloroquine, Didesethyl-chloroquine, Quinine and/or Doxycycline need to be assayed in the same study. See the appropriate validation report for volume and concentration information.

Generation of Calibration Standard and Quality Control Samples

The schemes for generating calibration standard and quality control (QC) samples for Doxycycline are provided in the following descriptions.

Calibration Standards: Calibration standards were generated by spiking with 128 µl of high working solution into 872 µl of blank dog plasma (Solution 1). Pipette 500 µl of solution 1 into 500 µl of blank dog plasma (Solution 2). Pipette

500 µl of solution 2 into 500 µl of blank dog plasma (Solution 3). Pipette 500 µl of solution 3 into 500 µl of blank dog plasma (Solution 4). Pipette 500 µl of solution 4 into 500 µl of blank dog plasma (Solution 5). Pipette 500 µl of solution 5 into 500 µl of blank dog plasma (Solution 6). Pipette 500 µl of solution 6 into 500 µl of blank dog plasma (Solution 7). Pipette 500 µl of solution 7 into 500 µl of blank dog plasma (Solution 8). Pipette 500 µl of solution 8 into 500 µl of blank dog plasma (Solution 9). These give final concentrations of 12800, 6400, 3200, 1600, 800, 400, 200, 100, and 50.0 ng/ml for Doxycycline. Vortex for 10 seconds.

Quality Control Samples: Quality control samples were generated by spiking with 64 µl of high working solution into 936 µl of blank dog plasma (High Solution). Pipette 250 µl of high solution into 750 µl of blank dog plasma (Medium solution). Pipette 250 µl of medium solution into 750 µl of blank dog plasma (Low solution). Pipette 250 µl of low solution into 750 µl of blank dog plasma (X Low solution). These give final concentrations of 6400, 1600, 400, and 100 ng/ml for Doxycycline. Vortex for 10 seconds.

SAMPLE PREPARATION PROCEDURE

1. Pipette 200 µl of blank dog plasma into a 13 x 100 silanized tube.
2. Spike standard curve samples with Doxycycline as described above.
3. Add 100 µl of methanol. Vortex for 1 minute.
4. Add 500 µl of internal standard working solution (500 ng/ml minocycline). For 0.0 sample, add 500 µl 100% CH_3CN. Vortex 1 minute.
5. Centrifuge for 5 minutes at 3000 rpm.
6. Transfer ~300 µl of supernatant to WISP vial and inject 1-2 µl onto the column.

Study Report No. 31

Short Validation of a Liquid Chromatographic/Mass Spectrometric/Mass Spectrometric (LC/MS/MS) Method for the Determination of Chloroquine (and its metabolites), Quinine, Doxycycline, Halofantrine (and its metabolite), Mefloquine, and WR 238,605 in Dog Plasma Samples
Part IV: Halofantrine (and metabolite) and WR 238,605

III. Methods

A. Materials

Test Compounds:	Halofantrine Halofantrine metabolite WR 238650
Drug Standards:	Halofantrine, Bottle number BM 01792, was obtained from WRAIR. Halofantrine metabolite, Bottle number BM 08577, was obtained from WRAIR. WR 238,605, Bottle number BM12562, was obtained from WRAIR.
Internal Standards:	WR 122455, Bottle number AX 26839, obtained from WRAIR. Verapamil, Lot number 68F-0758, was purchased from Sigma Chemical Co, St. Louis, MO.
Matrix:	Dog Plasma
Biological Matrix:	Control and standard curve dog plasma was obtained from Pel-Freez Biologicals, Rogers, AK. This matrix was used for daily preparation of standard and quality control samples. Prior to use, plasma was stored at -20°C. This material was found to be free of endogenous substances that would interfere with the quantitation of the drug or internal standard.
Sample Storage:	Temperature: Approximately -70°C

LC/MS/MS System

Detector:	API III PE-Sciex (Perkin-Elmer, Norwalk, CT)
Pump:	Shimadzu LC-10AD Pump (Shimadzu Scientific Instruments, Inc., Columbus, MD) or equivalent
Injector:	Waters Intelligent Sample Processor 717 Plus (Waters Associates, Milford, MA) or equivalent
Data Acquisition:	Macintosh Quadra 800 (Apple, Cupertino, CA) Mac Spec 3.3 Software (Perkin-Elmer, Norwalk, CT) RAD 2.4 Software (Perkin-Elmer, Norwalk, CT)
Data Reduction:	Internal standard method using peak area ratio (PAR). Weighted (1/y) linear regression of concentration (x-axis) vs. PAR (y-axis)

LC/MS/MS Conditions

Column:	Hypersil Silica, 3 µm particle size, 4.6 X 50 mm (Keystone Scientific, Inc., Bellefonte, PA) or equivalent
Column Temperature:	Room Temperature
Mobile Phase:	90% acetonitrile, 0.06% trifluoroacetic acid. The mobile phase was prepared by mixing 3600 ml of acetonitrile with 400 ml of water, adding 2.4 ml of trifluoroacetic acid to yield about 4 liters. The resulting solution was filtered through a 5 micron filter and degassed under vacuum prior to use.
Flow Rate:	1.2 ml/min. for halofantrine/metabolite 1.3 ml/min. WR 238,605
Sample Inlet Mode:	Heated Nebulizer
Ionization Mode:	APCI/Positive Ionization
Discharge Current:	+ 3 µA
Curtain Gas Flow Rate:	1.2 L/min. (N_2 = 99.999%)
Nebulizer Pressure:	80 psi (Ultra Zero Air)
Auxiliary Flow Rate:	2.0 L/min. (Ultra Zero Air)
CAD Gas:	250 x 10^{12} molecules/cm^2 (9.99% N_2/Ar)
Interface Heater Temp.:	55°C
Heated Nebulizer Temperature Controller:	450°C

Note: If necessary, the LC/MS/MS conditions can be slightly modified to optimize the system.

Mass Scanning Mode: MRM (Multiple Reaction Monitoring)

Halofantrine:	500 - 142 *m/z*
Halofantrine metabolite:	444 - 86 *m/z*
Halofantrine IS (WR122455):	428 - 410 *m/z*
WR 238,605:	464 - 86 *m/z*
WR 238,605 IS (Verapamil):	455 - 165 *m/z*

Assay Parameters

Volume of Plasma Required for Assay:	100 µl
Assay Ranges:	Halofantrine: 2.00 to 400 ng/ml Halofantrine metabolite: 2.00 to 400 ng/ml WR 238,605: 2.00 to 400 ng/ml
Minimum Reportable Concentrations:	Halofantrine: 2.00 ng/ml Halofantrine metabolite: 2.00 ng/ml WR 238,605: 2.00 ng/ml

Chemicals and Supplies

Chemical/Solvents	Grade	Supplier
Acetonitrile	HPLC	Fisher Scientific
Trifluoroacetic Acid	Reagent	Sigma Chemical
Water	Type I Reagent Grade	Nanopure, Barnstead
Methanol	Optima	Fisher Scientific

B. Analytical Method

Dog plasma samples (100 µl) were analyzed for Halofantrine, Halofantrine metabolite and WR 238,605 (Halofantrine and metabolite were analyzed in a separate run from WR 238,605) with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a silica column (4.6 x 50 mm, 3 µm particle size), 90% CH_3CN, 0.06% trifluoroacetic acid (TFA) mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (atmospheric pressure chemical ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of 100 µl of methanol, addition of verapamil/WR 122455 internal standard (IS) in acetonitrile, and transfer of the supernatant prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free dog plasma samples with known amounts of Halofantrine, Halofantrine metabolite, WR 238,605, and IS. Standard curve, QC and assay samples were prepared as described, then 3-5 µl aliquots were injected into the LC/MS/MS system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios of Halofantrine, Halofantrine metabolite and WR 238,605 to IS were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations and Halofantrine, Halofantrine metabolite and WR 238,605 to IS peak area ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the equations for the best straight lines ($y = mx + b$, where y = peak area ratio and x = Halofantrine, Halofantrine metabolite or WR 238,605 concentrations), and drug concentrations in assay samples were calculated by these equations from the Halofantrine, Halofantrine metabolite and WR 238,605 to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank dog plasma with Halofantrine, Halofantrine metabolite and WR 238,605.

Standard Curve and Control Solutions

STOCK SOLUTIONS: These solutions were stored in a 4°C refrigerator. Halofantrine, Halofantrine metabolite and WR 238,605 were protected from light.

Solution Type	Weight of Standard (mg)	Conversion Factor*	QS Volume (ml)	Solvent	Conc. (µg/ml)
Halofantrine Standard Curve	2.74	0.9321	25.54	CH_3OH	100
Halofantrine metabolite Standard Curve	2.76	0.9238	25.496	CH_3OH	100
Halofantrine Control	5.34	0.9321	50.280	CH_3OH	99.0
Halofantrine metabolite Control	5.42	0.9238	50.080	CH_3OH	100
WR 122455 (IS)	10.96	0.9214	10.099	CH_3OH	1000
WR 238,605 Standard Curve	6.27	0.7971	50.0	CH_3OH	0.100
WR 238,605 Control	6.40	0.7971	51.01	CH_3OH	0.100
Verapamil (IS)	10.04	1.00	10.04	50% CH_3OH	1.00

*= Molecular weights of halofantrine free base/halofantrine hydrochloride, WR 122455 free base/WR 122455 hydrochloride and WR 238,605 free base/WR 238,605 succinate.

WORKING SOLUTIONS. These solutions were stored in a 4°C refrigerator and protected against light. Halofantrine and Halofantrine metabolite stock solutions were combined into a single solution (high working solution, HWS) and diluted with methanol. Verapamil and WR 122455 stock solutions were combined into a single solution (HWS) and diluted with methanol. The low working solutions (LWS) were diluted from the HWS with methanol (except the internal standard LWS-with acetonitrile).

Solution Type	Volume Diluted (ml)	Conc. Diluted (µg/ml)	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve HWS					
[Halofantrine]	2.50	100	25.0	CH_3OH	10.0
[Halofantrine metabolite]	2.50	100			10.0
Standard Curve LWS					
[Halofantrine]	2.50	10.0	25.0	CH_3OH	1.00
[Halofantrine metabolite]		10.0			1.00
Control HWS					
[Halofantrine]	2.53	99.0	25.0	CH_3OH	10.0
[Halofantrine metabolite]	2.50	100			10.0
Control LWS					
[Halofantrine]	2.50	10.0	25.0	CH_3OH	1.00
[Halofantrine metabolite]		10.0			1.00
Standard Curve HWS					
[WR 238,605]	2.50	100	25.0	CH_3OH	10.0
Standard Curve LWS					
[WR 238,605]	2.50	10.0	25.0	CH_3OH	1.00
Control HWS					
[WR 238,605]	2.50	100	25.0	CH_3OH	10.0

Solution Type	Volume Diluted (ml)	Conc. Diluted (µg/ml)	QS Volume (ml)	Solvent	Conc. (µg/ml)
Control LWS					
[WR 238,605]	2.50	10.0	25.0	CH_3OH	1.00
Internal Standard HWS					
[Verapamil]	0.025	1000	25.0	CH_3OH	1.00
[WR 122455]	0.025	1000			1.00
Internal Standard LWS					
[Verapamil]	1.00	1.00	100	CH_3CH	0.010
[WR 122455]		1.00			0.010

Calibration Standards and Quality Control Samples

The scheme for generating calibration standard and quality control (QC) samples is provided in the following tables.

Calibration Standards: Calibration standards were generated by adding blank dog plasma and Halofantrine/Halofantrine metabolite and WR 238,605 HWS or LWS standard curve solutions to sample tubes to make 1 ml specimens. These specimens were divided into 100 µl aliquots for use as calibration standard samples with the Halofantrine, Halofantrine metabolite and WR 238,605 concentrations shown below.

Generation of Standard Curve Calibrators

Sample Number	Standard Solution Volume (of Each) (µl)	Dog Plasma Volume (µl)	Spiking Solution Conc. (of Each) (µg/ml)	Standard Compound Mass (of Each) (ng)	Standard Curve Sample Conc. (of Each) (ng/ml)
00*	0.00	1000	0	0	0
0**	0.00	1000	0	0	0
1	2.00	996	1.00	2.00	2.00
2	4.00	992	1.00	4.00	4.00
3	8.00	984	1.00	8.00	8.00
4	15.0	970	1.00	15.0	15.0
5	3.00	994	10.0	30.0	30.0
6	5.00	990	10.0	50.0	50.0
7	10.0	980	10.0	100	100
8	20.0	960	10.0	200	200
9	40.0	920	10.0	400	400

Quality Control Samples: QC samples were generated by adding blank dog plasma and Halofantrine/Halofantrine metabolite and WR 238,605 HWS or LWS standard curve solutions to sample tubes to make 1 ml specimens. These specimens were divided into 100 µl aliquots for use as QC samples with the

* Sample with no drug and no internal standard.

** Sample with no drug, but with internal standard.

Halofantrine, Halofantrine metabolite and WR 238,605 concentrations shown below.

Generation of Precision Quality Control Samples

Sample ID	Control Solution Volume (of Each) (µl)	Dog Plasma Volume (µl)	Spiking Solution Conc. (of Each) (µg/ml)	Standard Compound Mass (of Each) (ng)	QC Sample Conc. (of Each) (ng/ml)
X-Low	4.00	992	1.00	4.00	4.00
Low	20.0	960	1.00	20.0	20.0
Med.	8.00	984	10.0	80.0	80.0
High	32.0	936	10.0	320	320

SAMPLE PREPARATION PROCEDURE

1. Pipette 100 µl of dog plasma into a 13 x 100 silanized tube.
2. Spike standard curve samples with Halofantrine, Halofantrine metabolite and WR 238,605 as described above.
3. Add 100 µl of methanol. Vortex for 1 minute.
4. Add 300 µl of internal standard working solution (10.0 ng/ml verapamil and WR 122455) and vortex 1 minute.
5. Centrifuge for 5 minutes at 3000 rpm.
6. Transfer supernatant to WISP vial and inject 3-5 µl onto the column.

APPENDIX B

ANALYTICAL RESULTS

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
1695-pilot-0				*	*				
1695-pilot-0.25				793	809			*	
1695-pilot-0.5				1680	1010			7.82	
1695-pilot-1				2830	1400			84.7	
1695-pilot-2				2450	1460			195	
1695-pilot-4				1450	1040			285	
1695-pilot-6				1180	527			358	
1695-pilot-8				749	304			266	
1695-pilot-12				175	239			195	
1695-pilot-18				42.5	271			167	
1695-pilot-24				10.4	298			103	
1695-pilot-48				*	63.6			34.3	
1695-pilot-72				*	*			11.0	
1695-pilot-96				*	*			*	
1695-pilot-120				*	*			*	
1695-pilot-168				*	*			*	
1695-pilot-336				*	*				
1695-pilot-504				*	*				
1696-pilot-0				*	*				*
1696-pilot-0.5				*	*				333
1696-pilot-2				*	*				780
1696-pilot-4				*	*				831
1696-pilot-8				*	*				825
1696-pilot-10				*	*				776
1696-pilot-10.5				935	705				536
1696-pilot-11				1560	2200				574
1696-pilot-12				1490	2300				522
1696-pilot-13				1200	2030				544
1696-pilot-14				945	1940				545
1696-pilot-20				30.3	115				818
1696-pilot-28				*	76.3				818
1696-pilot-48				*	*				594
1696-pilot-72				*	*				494
1696-pilot-96				*	*				489
1696-pilot-120				*	*				467
1696-pilot-168				*	*				354
1696-pilot-336				*	*				158
1696-pilot-504				*	*				65.0

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
1697-pilot-0									*
1697-pilot-0.25									118
1697-pilot-0.5									267
1697-pilot-1									482
1697-pilot-2									598
1697-pilot-4									789
1697-pilot-6									928
1697-pilot-8									1020
1697-pilot-12									815
1697-pilot-18									792
1697-pilot-24									722
1697-pilot-48									589
1697-pilot-72									525
1697-pilot-96									468
1697-pilot-120									438
1697-pilot-168									339
1697-pilot-336									178
1697-pilot-504									88.8
1698-pilot-0	*	*	*						*
1698-pilot-0.5	*	*	*						56.2
1698-pilot-2	*	*	*						433
1698-pilot-4	*	*	*						535
1698-pilot-8	*	*	*						635
1698-pilot-9	26.4	*	*						689
1698-pilot-10	42.0	*	6.55						624
1698-pilot-11	25.9	*	4.02						679
1698-pilot-12	35.4	*	8.43						639
1698-pilot-13	40.6	*	11.3						770
1698-pilot-16	43.0	*	16.1						768
1698-pilot-20	28.1	*	17.5						767
1698-pilot-24	23.5	*	20.6						616
1698-pilot-48	9.24	*	53.0						446
1698-pilot-72	12.7	7.17	200						492
1698-pilot-96	*	*	11.3						317
1698-pilot-120	*	*	12.7						266
1698-pilot-168	*	*	12.7						152
1698-pilot-336	*	*	5.16						46.4
1698-pilot-504	*	*	*						20.1

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
1699-pilot-0	*	*	*						
1699-pilot-0.25	14.5	*	*						
1699-pilot-0.5	25.8	*	*						
1699-pilot-1	31.0	*	6.92						
1699-pilot-2	32.4	*	14.9						
1699-pilot-4	30.4	*	20.7						
1699-pilot-6	40.8	4.08	37.9						
1699-pilot-8	31.3	4.16	36.1						
1699-pilot-12	17.1	*	32.0						
1699-pilot-18	10.6	*	23.1						
1699-pilot-24	6.77	*	28.3						
1699-pilot-48	*	5.57	60.6						
1699-pilot-72	*	24.1	241						
1699-pilot-96	*	4.84	29.8						
1699-pilot-120	*	*	11.6						
1699-pilot-168	*	5.56	27.5						
1699-pilot-336	*	*	6.23						
1699-pilot-504	*	6.12	14.7						
1785-pilot-0						*	*		
1785-pilot-0.25						88.1	*		
1785-pilot-0.5						234	4.45		
1785-pilot-1						350	14.0		
1785-pilot-2						*	*		608
1785-pilot-4						447	64.1		
1785-pilot-6						433	90.9		
1785-pilot-8						352	103		
1785-pilot-12						197	96.5		
1785-pilot-18						1570	109		
1785-pilot-24						633	109		
1785-pilot-48						128	78.4		
1785-pilot-72						79.1	57.4		
1785-pilot-96						54.6	35.5		
1785-pilot-120						50.3	21.6		
1785-pilot-168						40.0	10.6		
1785-pilot-336						23.5	2.81		
1785-pilot-504						15.1	*		

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
1786-pilot-0						*	*		*
1786-pilot-0.5						*	*		127
1786-pilot-2						530	34.9		*
1786-pilot-4						2.37	*		706
1786-pilot-8						*	*		737
1786-pilot-9						1330	15.3		823
1786-pilot-10						2560	38.6		739
1786-pilot-11						2330	54.7		820
1786-pilot-12						2410	69.1		732
1786-pilot-13						1930	76.7		729
1786-pilot-16						2100	114		721
1786-pilot-20						1140	120		553
1786-pilot-24						848	131		591
1786-pilot-48						213	87.1		418
1786-pilot-96						98.4	51.1		342
1786-pilot-120						74.4	37.8		289
1786-pilot-168						56.6	22.5		257
1786-pilot-336						19.9	3.39		70.5
1786-pilot-504						13.4	2.17		23.7
1972-H-0				*	*				
1972-H-0.5				2980	474				
1972-H-1				2600	452				
1972-H-2				2860	435				
1972-H-4				4050	410				
1972-H-6				1610	387				
1972-H-7				1470	353				
1972-H-8				862	386				
1972-H-10				412	412				
1972-H-12				235	395				
1972-H-18				68.9	151				
1972-H-24				131	*				
1972-H-48				*	*				
1972-H-72				*	*				
1972-H-96				*	*				
1972-H-120				*	*				
1972-H-168				*	*				

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
1973-I-0				*	*				*
1973-I-0.5				12.4	*				44.8
1973-I-1				*	*				138
1973-I-2				*	*				332
1973-I-4				*	*				430
1973-I-6				*	*				430
1973-I-7				2500	1630				336
1973-I-8				2110	1620				275
1973-I-10				1480	1040				282
1973-I-12				918	999				291
1973-I-18				59.8	252				573
1973-I-24				41.5	122				481
1973-I-48				*	65.7				331
1973-I-72				*	*				368
1973-I-96				*	*				356
1973-I-120				*	*				222
1973-I-168				*	*				217
1973-I-336				*	*				68.7
1973-I-504				*	*				13.1
1974-I-0				*	*				*
1974-I-0.5				*	*				89.1
1974-I-1				*	*				180
1974-I-2				*	*				284
1974-I-4				*	*				356
1974-I-6				*	*				358
1974-I-7				2720	1130				224
1974-I-8				1970	916				199
1974-I-10				1730	825				199
1974-I-12				1230	769				209
1974-I-18				281	178				395
1974-I-24				45.6	85.4				430
1974-I-48				*	*				266
1974-I-72				*	*				278
1974-I-96				*	*				300
1974-I-120				*	*				162
1974-I-168				*	*				113
1974-I-336				*	*				22.8
1974-I-504				*	*				5.10

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
1975-I-0				*	*				*
1975-I-0.5				*	*				163
1975-I-1				*	*				252
1975-I-2				*	*				486
1975-I-4				*	*				505
1975-I-6				*	*				518
1975-I-7				2660	2410				386
1975-I-8				2380	2190				378
1975-I-10				1830	1550				375
1975-I-12				1040	1620				352
1975-I-18				60.4	236				591
1975-I-24				13.0	64.9				611
1975-I-48				*	*				329
1975-I-72				*	*				356
1975-I-96				*	*				280
1975-I-120				*	*				177
1975-I-168				*	*				94.4
1975-I-336				*	*				9.62
1975-I-504				*	*				*
1976-I-0				*	*				*
1976-I-0.5				*	*				72.0
1976-I-1				*	*				218
1976-I-2				*	*				384
1976-I-4				*	*				468
1976-I-6				*	*				464
1976-I-7				1890	1330				464
1976-I-8				1980	1760				343
1976-I-10				1550	266				370
1976-I-12				1090	1310				326
1976-I-18				104	1180				424
1976-I-24				23.3	78.2				469
1976-I-48				*	*				361
1976-I-72				*	*				315
1976-I-96				*	*				364
1976-I-120				*	*				213
1976-I-168				*	*				187
1976-I-336				*	*				56.9
1976-I-504				*	*				15.1

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
1977-H-0				*	*				
1977-H-0.5				4180	1280				
1977-H-1				4060	1820				
1977-H-2				3060	1690				
1977-H-4				1510	1510				
1977-H-6				1010	1320				
1977-H-7				683	1160				
1977-H-8				475	1040				
1977-H-10				136	1040				
1977-H-12				40.6	901				
1977-H-18				10.1	257				
1977-H-24				*	82.6				
1977-H-48				*	*				
1977-H-72				*	*				
1977-H-96				*	*				
1977-H-120				*	*				
1977-H-168				*	*				
1978-H-0				*	*				
1978-H-0.5				3090	1510				
1978-H-1				1860	1530				
1978-H-2				1510	1450				
1978-H-4				1150	927				
1978-H-6				893	1010				
1978-H-7				784	859				
1978-H-8				677	823				
1978-H-10				558	629				
1978-H-12				310	629				
1978-H-18				26.8	171				
1978-H-24				*	*				
1978-H-48				*	*				
1978-H-72				*	*				
1978-H-96				*	*				
1978-H-120				*	*				
1978-H-168				*	*				

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
1979-F-0						*	*		
1979-F-0.5						14.2	*		
1979-F-1						43.6	*		
1979-F-2						146	3.91		
1979-F-4						529	21.2		
1979-F-6						577	60.4		
1979-F-8						359	65.7		
1979-F-10						259	75.2		
1979-F-12						228	85.3		
1979-F-18						189	83.7		
1979-F-24						314	67.1		
1979-F-48						70.6	45.1		
1979-F-72						49.9	35.0		
1979-F-96						42.6	30.1		
1979-F-120						32.6	14.3		
1979-F-168						22.3	7.21		
1979-F-336						8.52	*		
1979-F-504						5.22	*		
1980-F-0						*	*		
1980-F-0.5						79.8	*		
1980-F-1						240	4.43		
1980-F-2						275	12.9		
1980-F-4						222	24.1		
1980-F-6						263	42.6		
1980-F-8						200	43.1		
1980-F-10						157	53.7		
1980-F-12						103	47.6		
1980-F-18						92.3	40.7		
1980-F-24						46.5	23.7		
1980-F-48						12.3	11.7		
1980-F-72						7.48	5.21		
1980-F-96						5.88	*		
1980-F-120						7.40	*		
1980-F-168						4.37	*		
1980-F-336						*	*		
1980-F-504						*	*		

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
1981-H-0				*	*				
1981-H-0.5				4550	546				
1981-H-1				3990	809				
1981-H-2				3890	931				
1981-H-4				3200	750				
1981-H-6				1840	888				
1981-H-7				1650	697				
1981-H-8				1520	704				
1981-H-10				1250	733				
1981-H-12				937	678				
1981-H-18				167	195				
1981-H-24				49.3	*				
1981-H-48				*	*				
1981-H-72				*	*				
1981-H-96				*	*				
1981-H-120				*	*				
1981-H-168				*	*				
1982-F-0						*	*		
1982-F-0.5						*	*		
1982-F-1						23.9	*		
1982-F-2						225	8.26		
1982-F-4						219	36.3		
1982-F-6						391	86.2		
1982-F-8						222	84.8		
1982-F-10						143	95.8		
1982-F-12						102	90.9		
1982-F-18						873	86.0		
1982-F-24						308	105		
1982-F-48						50.7	76.1		
1982-F-72						23.0	48.9		
1982-F-96						16.8	31.8		
1982-F-120						14.0	13.3		
1982-F-168						8.85	5.13		
1982-F-336						3.59	*		
1982-F-504						2.63	*		

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
1983-F-0						*	*		
1983-F-0.5						4.76	*		
1983-F-1						11.2	*		
1983-F-2						197	3.73		
1983-F-4						244	16.4		
1983-F-6						323	35.6		
1983-F-8						272	35.0		
1983-F-10						248	39.6		
1983-F-12						189	37.4		
1983-F-18						498	40.9		
1983-F-24						261	29.4		
1983-F-48						82.9	21.4		
1983-F-72						46.3	9.52		
1983-F-96						44.0	5.19		
1983-F-120						43.7	3.36		
1983-F-168						31.9	*		
1983-F-336						18.0	*		
1983-F-504						11.7	*		
1984-I-0				*	*				*
1984-I-0.5				*	*				37.8
1984-I-1				*	*				148
1984-I-2				*	*				331
1984-I-4				*	*				431
1984-I-6				*	*				483
1984-I-7				2200	1680				446
1984-I-8				1860	1350				373
1984-I-10				1130	1390				323
1984-I-12				666	1250				307
1984-I-18				69.2	183				451
1984-I-24				13.4	78.0				468
1984-I-48				*	*				331
1984-I-72				*	*				366
1984-I-96				*	*				386
1984-I-120				*	*				238
1984-I-168				*	*				205
1984-I-336				*	*				80.8
1984-I-504				*	*				15.5

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
1985-G0						*	*		*
1985-G-0.5						25.9	*		259
1985-G-1						158	4.35		504
1985-G-2						309	16.0		665
1985-G-4						489	38.4		689
1985-G-6						396	63.8		779
1985-G-8						332	76.8		779
1985-G-10						268	94.6		699
1985-G-12						260	104		777
1985-G-18						485	94.1		743
1985-G-24						413	85.7		562
1985-G-48						63.6	59.8		523
1985-G-72						29.0	30.0		418
1985-G-96						24.9	14.9		388
1985-G-120						24.5	7.14		334
1985-G-168						16.0	3.54		219
1985-G-336						7.72	*		57.7
1985-G-504						5.32	*		24.8
1986-G-0						*	*		*
1986-G-0.5						110	*		420
1986-G-1						220	6.17		493
1986-G-2						311	19.0		673
1986-G-4						217	31.0		788
1986-G-6						198	51.3		646
1986-G-8						180	53.1		751
1986-G-10						109	59.5		816
1986-G-12						78.7	58.5		814
1986-G-18						286	59.0		686
1986-G-24						142	46.4		593
1986-G-48						19.7	26.1		513
1986-G-72						12.2	14.5		389
1986-G-96						10.3	8.74		393
1986-G-120						9.11	4.30		306
1986-G-168						5.76	*		212
1986-G-336						*	*		53.7
1986-G-504						*	*		18.4

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
1987-G-0						*	*		*
1987-G-0.5						81.9	*		211
1987-G-1						437	5.17		502
1987-G-2						567	15.0		654
1987-G-4						505	26.2		717
1987-G-6						337	57.0		813
1987-G-8						346	60.2		690
1987-G-10						226	67.7		688
1987-G-12						151	65.0		767
1987-G-18						113	55.0		736
1987-G-24						63.5	35.5		579
1987-G-48						29.7	29.8		574
1987-G-72						19.8	16.3		413
1987-G-96						16.5	10.4		429
1987-G-120						13.1	3.84		292
1987-G-168						8.15	*		190
1987-G-336						3.43	*		28.0
1987-G-504						*	*		7.32
1988-H-0				*	57.6				
1988-H-0.5				1840	1220				
1988-H-1				1860	1470				
1988-H-2				1450	1300				
1988-H-4				954	1070				
1988-H-6				593	931				
1988-H-7				392	797				
1988-H-8				259	844				
1988-H-10				78.4	738				
1988-H-12				26.8	728				
1988-H-18				*	231				
1988-H-24				*	171				
1988-H-48				*	67.1				
1988-H-72				*	*				
1988-H-96				*	*				
1988-H-120				*	*				
1988-H-168				*	*				

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
1989-G-0						*	*		*
1989-G-0.5						2.40	*		13.0
1989-G-1						69.5	*		144
1989-G-2(100ul)						112	11.2		237
1989-G-4						133	17.0		378
1989-G-6						309	60.3		715
1989-G-8						468	63.0		773
1989-G-10						372	94.9		846
1989-G-12						309	88.6		742
1989-G-18						582	88.1		712
1989-G-24						223	67.1		564
1989-G-48						56.0	42.3		416
1989-G-72						30.8	21.0		355
1989-G-96						26.7	15.4		358
1989-G-120						20.9	6.80		261
1989-G-168						14.0	3.93		180
1989-G-336						5.08	*		33.4
1989-G-504						*	*		4.96
1990-G-0						*	*		*
1990-G-0.5						18.4	*		76.4
1990-G-1						124	3.86		275
1990-G-2						170	10.5		534
1990-G-4						281	28.9		763
1990-G-6						368	54.7		771
1990-G-8						280	58.3		772
1990-G-10						182	66.1		829
1990-G-12						140	67.1		864
1990-G-18						ns	ns		ns
1990-G-24						211	54.2		626
1990-G-48						48.3	32.7		602
1990-G-72						32.8	17.5		446
1990-G-96						29.0	12.0		494
1990-G-120						21.9	5.90		387
1990-G-168						15.3	3.91		265
1990-G-336						7.05	*		80.8
1990-G-504						4.21	*		31.5

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
1991-I-0				*	*				*
1991-I-0.5				*	*				111
1991-I-1				*	*				250
1991-I-2				*	*				382
1991-I-4				*	*				516
1991-I-6				*	*				502
1991-I-7				3380	891				365
1991-I-8				3350	1110				287
1991-I-10				2490	1080				224
1991-I-12				1560	1240				237
1991-I-18				108	239				628
1991-I-24				32.2	76.5				619
1991-I-48				*	*				379
1991-I-72				*	*				373
1991-I-96				*	*				393
1991-I-120				*	*				232
1991-I-168				*	*				181
1991-I-336				*	*				62.2
1991-I-504				*	*				17.5
1992-H-0				*	*				
1992-H-0.5				1470	815				
1992-H-1				2710	1940				
1992-H-2				1980	1970				
1992-H-4				1650	1600				
1992-H-6				1270	1400				
1992-H-7				1130	1350				
1992-H-8				898	1140				
1992-H-10				590	1180				
1992-H-12				272	972				
1992-H-18				29.2	253				
1992-H-24				13.8	169				
1992-H-48				*	64.9				
1992-H-72				*	*				
1992-H-96				*	*				
1992-H-120				*	54.2				
1992-H-168				*	*				

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
1993-F-0						*	*		
1993-F-0.5						195	3.05		
1993-F-1						695	12.7		
1993-F-2						580	23.4		
1993-F-4						374	51.6		
1993-F-6						373	87.3		
1993-F-8						255	86.3		
1993-F-10						170	93.9		
1993-F-12						136	92.6		
1993-F-18						792	90.2		
1993-F-24						21.0	9.86		
1993-F-48						379	85.9		
1993-F-72						61.9	54.1		
1993-F-96						31.7	30.0		
1993-F-120						23.9	18.7		
1993-F-168						11.9	3.84		
1993-F-336						5.07	*		
1993-F-504						*	*		
1994-F-0						*	*		
1994-F-0.5						47.5	*		
1994-F-1						201	5.35		
1994-F-2						313	25.3		
1994-F-4						449	60.4		
1994-F-6						948	163		
1994-F-8						887	155		
1994-F-10						754	200		
1994-F-12						641	182		
1994-F-18						287	140		
1994-F-24						175	104		
1994-F-48						56.0	77.6		
1994-F-72						37.5	53.7		
1994-F-96						15.3	28.0		
1994-F-120						25.3	18.5		
1994-F-168						14.0	7.68		
1994-F-336						5.97	*		
1994-F-504						2.17	*		

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
1995-G-0						*	*		*
1995-G-0.5						52.1	*		160
1995-G-1						168	4.97		305
1995-G-2						230	15.2		587
1995-G-4						307	32.3		651
1995-G-6						569	76.6		859
1995-G-8						314	79.6		821
1995-G-10						222	92.9		776
1995-G-12						170	84.5		803
1995-G-18						573	111		810
1995-G-24						1180	88.6		590
1995-G-48						203	61.5		587
1995-G-72						110	37.4		518
1995-G-96						86.7	29.9		517
1995-G-120						77.4	15.6		389
1995-G-168						46.5	10.9		343
1995-G-336						26.5	3.23		76.9
1995-G-504						12.1	*		12.4
2006-C-0								*	*
2006-C-0.5								*	336
2006-C-1								*	526
2006-C-2								*	610
2006-C-4								878	724
2006-C-6								803	665
2006-C-8								759	602
2006-C-10								776	770
2006-C-12								765	569
2006-C-18								861	522
2006-C-24								547	541
2006-C-48								254	392
2006-C-72								91.9	337
2006-C-96								42.9	246
2006-C-120								19.5	222
2006-C-168								*	127
2006-C-336								*	15.8
2006-C-504								*	*

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
2007-C-0								*	*
2007-C-0.5								*	70.8
2007-C-1								*	142
2007-C-2								*	226
2007-C-4								929	277
2007-C-6								1090	304
2007-C-8								1170	303
2007-C-10								1190	324
2007-C-12								1090	389
2007-C-18								994	351
2007-C-24								878	240
2007-C-48								393	219
2007-C-72								244	212
2007-C-96								122	159
2007-C-120								85.2	165
2007-C-168								28.4	115
2007-C-336								*	46.1
2007-C-504								*	20.2
2008-B-0								*	
2008-B-0.5								9.96	
2008-B-1								184	
2008-B-2								464	
2008-B-4								738	
2008-B-6								793	
2008-B-8								666	
2008-B-10								572	
2008-B-12								594	
2008-B-18								363	
2008-B-24								234	
2008-B-48								87.5	
2008-B-72								32.3	
2008-B-96								8.62	
2008-B-120								*	
2008-B-168								*	
2008-B-336								*	
2008-B-504								*	

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
2009-B-0								*	
2009-B-0.5								*	
2009-B-1								22.8	
2009-B-2								338	
2009-B-4								579	
2009-B-6								630	
2009-B-8								639	
2009-B-10								641	
2009-B-12								599	
2009-B-18								309	
2009-B-24								241	
2009-B-48								103	
2009-B-72								39.7	
2009-B-96								12.8	
2009-B-120								*	
2009-B-168								*	
2009-B-336								*	
2009-B-504								*	
2010-A-0									*
2010-A-0.5									116
2010-A-1									354
2010-A-2									517
2010-A-4									526
2010-A-6									651
2010-A-8									703
2010-A-10									695
2010-A-12									638
2010-A-18									580
2010-A-24									372
2010-A-48									410
2010-A-72									433
2010-A-96									393
2010-A-120									343
2010-A-168									255
2010-A-336									150
2010-A-504									79.3

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
2011-A-0									*
2011-A-0.5									311
2011-A-1									605
2011-A-2									759
2011-A-4									704
2011-A-6									1000
2011-A-8									998
2011-A-10									982
2011-A-12									924
2011-A-18									822
2011-A-24									604
2011-A-48									590
2011-A-72									587
2011-A-96									499
2011-A-120									346
2011-A-168									283
2011-A-336									89.9
2011-A-504									33.4
2012-C-0								*	*
2012-C-0.5								*	26.1
2012-C-1								*	375
2012-C-2								*	603
2012-C-4								74.9	1020
2012-C-6								875	890
2012-C-8								860	887
2012-C-10								934	1030
2012-C-12								824	923
2012-C-18								636	915
2012-C-24								972	634
2012-C-48								745	571
2012-C-72								325	538
2012-C-96								148	434
2012-C-120								59.5	418
2012-C-168								17.1	342
2012-C-336								*	96.8
2012-C-504								*	28.5

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
2013-A-0									*
2013-A-0.5									80.1
2013-A-1									428
2013-A-2									674
2013-A-4									923
2013-A-6									861
2013-A-8									955
2013-A-10									NS
2013-A-12									868
2013-A-18									702
2013-A-24									728
2013-A-48									602
2013-A-72									628
2013-A-96									444
2013-A-120									314
2013-A-168									187
2013-A-336									38.9
2013-A-504									14.6
2014-B-0								122	
2014-B-0.5								152	
2014-B-1								635	
2014-B-2								1060	
2014-B-4								1200	
2014-B-6								1130	
2014-B-8								1270	
2014-B-10								902	
2014-B-12								1040	
2014-B-18								1180	
2014-B-24								1290	
2014-B-48								805	
2014-B-72								528	
2014-B-96								224	
2014-B-120								101	
2014-B-168								38.7	
2014-B-336								*	
2014-B-504								*	

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
2015-E-0	*	*	*						*
2015-E-0.5	8.78	*	*						79.9
2015-E-1	90.9	*	11.1						361
2015-E-2	56.9	*	19.0						541
2015-E-4	138	7.04	125						565
2015-E-6	69.6	6.75	70.6						719
2015-E-8	127	17.5	256						776
2015-E-10	92.2	18.9	233						764
2015-E-12	54.9	13.5	145						706
2015-E-18	55.9	25.0	324						754
2015-E-24	45.3	37.8	500						604
2015-E-48	19.1	33.6	420						472
2015-E-72	8.01	36.6	360						436
2015-E-96	*	25.1	202						291
2015-E-120	*	12.9	96.1						251
2015-E-168	*	35.8	259						209
2015-E-336	*	12.7	48.2						72.6
2015-E-504	*	8.36	21.2						24.5
2017-A-0									*
2017-A-0.5									73.1
2017-A-1									130
2017-A-2									183
2017-A-4									305
2017-A-6									373
2017-A-8									370
2017-A-10									443
2017-A-12									338
2017-A-18									343
2017-A-24									249
2017-A-48									157
2017-A-72									145
2017-A-96									114
2017-A-120									67.3
2017-A-168									41.9
2017-A-336									5.05
2017-A-504									*

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
2018-D-0	*	*	*						
2018-D-0.5	37.8	*	*						
2018-D-1	77.0	*	11.3						
2018-D-2	75.9	*	28.9						
2018-D-4	116	*	98.3						
2018-D-6	85.7	6.50	117						
2018-D-8	151	16.6	276						
2018-D-10	78.9	15.5	194						
2018-D-12	85.2	25.4	345						
2018-D-18	75.8	34.0	465						
2018-D-24	46.9	42.7	519						
2018-D-48	13.4	30.9	344						
2018-D-72	6.85	31.1	287						
2018-D-96	*	20.9	169						
2018-D-120	*	14.3	130						
2018-D-168	*	12.2	91.0						
2018-D-336	*	11.2	58.4						
2018-D-504	*	7.38	32.0						
2019-A-0									*
2019-A-0.5									112
2019-A-1									213
2019-A-2									303
2019-A-4									413
2019-A-6									526
2019-A-8									646
2019-A-10									608
2019-A-12									528
2019-A-18									436
2019-A-24									309
2019-A-48									234
2019-A-72									305
2019-A-96									263
2019-A-120									158
2019-A-168									143
2019-A-336									51.6
2019-A-504									20.0

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
2020-C-0								*	*
2020-C-0.5								*	130
2020-C-1								*	332
2020-C-2								*	499
2020-C-4								586	566
2020-C-6								896	609
2020-C-8								821	579
2020-C-10								878	658
2020-C-12								638	637
2020-C-18								876	789
2020-C-24								473	454
2020-C-48								289	429
2020-C-72								215	487
2020-C-96								97.3	372
2020-C-120								57.0	350
2020-C-168								20.5	279
2020-C-336								*	133
2020-C-504								*	70.5
2021-D-0	*	*	*						
2021-D-0.5	65.2	*	*						
2021-D-1	81.6	*	6.97						
2021-D-2	125	*	19.0						
2021-D-4	250	*	89.1						
2021-D-6	102	*	46.7						
2021-D-8	216	*	153						
2021-D-10	118	*	102						
2021-D-12	122	4.33	138						
2021-D-18	166	15.8	426						
2021-D-24	122	17.8	427						
2021-D-48	27.7	13.8	280						
2021-D-72	19.0	19.4	361						
2021-D-96	10.1	18.2	279						
2021-D-120	5.46	11.9	181						
2021-D-168	*	12.0	139						
2021-D-336	*	7.51	52.1						
2021-D-504	*	4.96	30.5						

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
2022-D-0	*	*	*						
2022-D-0.5	59.7	*	4.81						
2022-D-1	42.7	*	6.88						
2022-D-2	65.8	*	18.7						
2022-D-4	116	*	68.0						
2022-D-6	60.9	*	43.5						
2022-D-8	138	7.55	168						
2022-D-10	126	12.6	240						
2022-D-12	121	18.1	347						
2022-D-18	58.6	19.8	300						
2022-D-24	67.6	32.1	521						
2022-D-48	8.29	10.0	133						
2022-D-72	4.18	10.5	111						
2022-D-96	*	14.3	95.4						
2022-D-120	*	*	27.6						
2022-D-168	*	4.91	34.3						
2022-D-336	*	*	15.1						
2022-D-504	*	*	10.7						
2023-B-0								*	
2023-B-0.5								55.8	
2023-B-1								503	
2023-B-2								996	
2023-B-4								1210	
2023-B-6								967	
2023-B-8								860	
2023-B-10								759	
2023-B-12								832	
2023-B-18								643	
2023-B-24								412	
2023-B-48								177	
2023-B-72								63.1	
2023-B-96								32.1	
2023-B-120								13.0	
2023-B-168								*	
2023-B-336								*	
2023-B-504								*	

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
2024-D-0	*	*	*						
2024-D-0.5	40.3	*	*						
2024-D-1	53.2	*	6.30						
2024-D-2	92.1	*	28.0						
2024-D-4	90.8	*	68.8						
2024-D-6	38.5	*	57.0						
2024-D-8	76.6	4.86	177						
2024-D-10	47.0	5.59	146						
2024-D-12	63.3	14.9	347						
2024-D-18	37.3	15.2	358						
2024-D-24	21.7	14.3	309						
2024-D-48	5.51	11.0	179						
2024-D-72	*	10.5	120						
2024-D-96	*	8.83	86.9						
2024-D-120	*	4.33	42.2						
2024-D-168	*	8.51	61.9						
2024-D-336	*	8.03	39.7						
2024-D-504	*	4.07	18.6						
2025-E-0	*	*	*						*
2025-E-0.5	67.6	*	*						223
2025-E-1	105	*	9.63						755
2025-E-2	163	*	40.5						783
2025-E-4	214	*	119						933
2025-E-6	77.3	*	43.9						995
2025-E-8	190	9.78	226						1050
2025-E-10	79.4	6.49	117						1300
2025-E-12	92.8	23.0	443						1180
2025-E-18	61.8	7.63	104						1000
2025-E-24	62.8	28.0	477						793
2025-E-48	18.3	21.0	305						691
2025-E-72	11.9	28.8	332						657
2025-E-96	4.74	18.1	167						493
2025-E-120	*	12.0	92.2						450
2025-E-168	*	7.55	53.6						370
2025-E-336	*	13.2	64.8						125
2025-E-504	*	7.68	29.9						37.3

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
2026-B-0								*	
2026-B-0.5								*	
2026-B-1								*	
2026-B-2								327	
2026-B-4								770	
2026-B-6								914	
2026-B-8								969	
2026-B-10								981	
2026-B-12								864	
2026-B-18								912	
2026-B-24								711	
2026-B-48								453	
2026-B-72								299	
2026-B-96								166	
2026-B-120								119	
2026-B-168								44.4	
2026-B-336								*	
2026-B-504								*	
2027-D-0	*	*	*						
2027-D-0.5	62.5	*	5.54						
2027-D-1	78.5	*	14.1						
2027-D-2	91.5	*	29.4						
2027-D-4	84.4	*	63.4						
2027-D-6	50.9	*	49.4						
2027-D-8	120	11.0	218						
2027-D-10	59.2	10.2	139						
2027-D-12	42.0	6.56	91.5						
2027-D-18	60.2	21.2	350						
2027-D-24	48.0	33.6	492						
2027-D-48	7.28	14.5	186						
2027-D-72	4.19	19.9	218						
2027-D-96	*	16.1	144						
2027-D-120	*	5.89	60.3						
2027-D-168	*	7.76	57.1						
2027-D-336	*	5.40	22.5						
2027-D-504	*	*	8.27						

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
2028-E-0	*	*	*						*
2028-E-0.5	47.8	*	*						307
2028-E-1	92.4	*	9.26						792
2028-E-2	146	*	29.8						814
2028-E-4	166	*	82.0						737
2028-E-6	89.1	*	41.2						851
2028-E-8	103	5.01	102						895
2028-E-10	108	4.78	104						972
2028-E-12	44.3	*	41.2						1060
2028-E-18	54.1	8.27	176						895
2028-E-24	12.9	8.62	122						641
2028-E-48	49.6	13.3	256						461
2028-E-72	6.64	8.60	107						533
2028-E-96	4.12	6.36	74.9						418
2028-E-120	*	8.88	92.0						338
2028-E-168	*	10.0	75.1						296
2028-E-336	*	6.42	44.8						168
2028-E-504	*	*	13.7						57.8
2029-C-0								*	*
2029-C-0.5								*	91.1
2029-C-1								*	175
2029-C-2								*	306
2029-C-4								698	413
2029-C-6								968	405
2029-C-8								1100	380
2029-C-10								1100	428
2029-C-12								966	464
2029-C-18								867	423
2029-C-24								669	294
2029-C-48								375	250
2029-C-72								267	250
2029-C-96								143	209
2029-C-120								107	238
2029-C-168								41.9	160
2029-C-336								*	61.3
2029-C-504								*	26.9

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
2030-D-0	*	*	*						
2030-D-0.5	95.8	*	6.45						
2030-D-1	88.4	*	9.96						
2030-D-2	130	*	27.7						
2030-D-4	142	*	63.7						
2030-D-6	69.9	*	44.1						
2030-D-8	207	5.08	181						
2030-D-10	89.6	*	97.1						
2030-D-12	122	10.3	232						
2030-D-18	132	11.3	324						
2030-D-24	71.9	15.2	311						
2030-D-48	18.6	9.34	191						
2030-D-72	10.3	16.4	255						
2030-D-96	*	8.61	120						
2030-D-120	*	4.64	75.0						
2030-D-168	*	9.40	83.3						
2030-D-336	*	8.11	50.1						
2030-D-504	*	*	17.4						
2031-A-0									*
2031-A-0.5									56.6
2031-A-1									220
2031-A-2									265
2031-A-4									390
2031-A-6									584
2031-A-8									681
2031-A-10									689
2031-A-12									676
2031-A-18									620
2031-A-24									562
2031-A-48									398
2031-A-72									374
2031-A-96									271
2031-A-120									211
2031-A-168									156
2031-A-336									34.7
2031-A-504									11.2

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
2032-C-0								*	*
2032-C-0.5								*	26.7
2032-C-1								*	269
2032-C-2								*	513
2032-C-4								85.8	748
2032-C-6								877	760
2032-C-8								911	864
2032-C-10								988	958
2032-C-12								938	961
2032-C-18								787	954
2032-C-24								1090	706
2032-C-48								629	609
2032-C-72								246	578
2032-C-96								81.4	368
2032-C-120								48	459
2032-C-168								8.88	349
2032-C-336								*	102
2032-C-504								*	31.0
2033-E-0	*	*	*						*
2033-E-0.5	91.6	*	*						157
2033-E-1	83.2	*	12.7						616
2033-E-2	76.6	*	26.5						669
2033-E-4	143	*	116						637
2033-E-6	123	5.35	188						743
2033-E-8	139	9.14	286						750
2033-E-10	47.6	*	68.3						925
2033-E-12	43.7	4.12	67.2						829
2033-E-18	46.2	14.1	358						676
2033-E-24	26.2	14.7	321						701
2033-E-48	6.55	12.4	195						454
2033-E-72	4.30	13.5	170						349
2033-E-96	*	12.7	131						248
2033-E-120	*	11.7	117						218
2033-E-168	*	13.3	132						153
2033-E-336	*	7.27	40.4						33.2
2033-E-504	*	*	17.2						8.41

* = below assay sensitivity.

TABLE 1: ANALYTICAL RESULTS: Multiple Drugs in Dog Plasma, Analytical Report WR5/P 95-3

Sample ID-Group-Hour	Chloro-quine (ng/ml)	Dides-ethylC (ng/ml)	Monodes-ethylC (ng/ml)	Quinine (ng/ml)	Doxy-cycline (ng/ml)	Halo-fantrine (ng/ml)	Halo. Metab. (ng/ml)	Meflo-quine (ng/ml)	WR 238605 (ng/ml)
2034-E-0	*	*	*						*
2034-E-0.5	55.8	*	*						77.4
2034-E-1	152	*	14.3						668
2034-E-2	148	*	38.9						838
2034-E-4	210	*	139						944
2034-E-6	134	5.74	156						1180
2034-E-8	182	13.4	274						1100
2034-E-10	176	*	116						1420
2034-E-12	105	19.6	319						1330
2034-E-18	111	55.9	797						1320
2034-E-24	63.7	56.6	752						895
2034-E-48	19.4	43.7	501						590
2034-E-72	9.12	58	522						649
2034-E-96	*	26.8	280						490
2034-E-120	*	31.6	335						425
2034-E-168	*	19.4	151						340
2034-E-336	*	7.23	44.4						151
2034-E-504	*	4.39	33.2						41.5
2035-E-0	*	*	*						*
2035-E-0.5	12.1	*	*						74.9
2035-E-1	78.4	*	*						447
2035-E-2	156	*	19.3						721
2035-E-4	186	*	53.8						895
2035-E-6	164	*	75.8						923
2035-E-8	214	*	130						1190
2035-E-10	212	18.5	447						1370
2035-E-12	121	*	106						1450
2035-E-18	159	10.1	362						1070
2035-E-24	142	12.2	467						1050
2035-E-48	38.8	6.87	218						626
2035-E-72	21.4	14.5	324						682
2035-E-96	10.4	13.8	254						588
2035-E-120	4.84	9.25	145						449
2035-E-168	*	10.8	168						390
2035-E-336	*	4.71	46.5						162
2035-E-504	*	4.59	36.9						51.2

* = below assay sensitivity.